An Investigation of Cytochrome P450 Function in Human Cell Lines for Potential Use In a Bioartificial Liver Device

A thesis submitted for the degree of Doctor of Philosophy (PhD)

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2010

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Declaration

I, Amy Thomas confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Bioartificial Liver Devices (BAL) could provide hepatic function in patients with liver failure awaiting transplantation or liver regeneration. Certain BALs use human hepatoblastoma-derived HepG2 cells that can perform many hepatocyte specific functions, however they cannot adequately detoxify xenobiotics via Cytochrome P450 (CYP) mediated pathways.

The aim of this thesis was to identify and develop a cell source able to provide CYP function in a BAL, and focused on CYP1A2, since its inducible function has been well established in HepG2 cells and CYP3A due its large contribution to hepatic CYP expression and function.

Firstly, gold standard comparators of freshly isolated primary human hepatocytes (PHH) were established. The influence of BAL culture conditions on CYP function were evaluated, including chemical induction, culture media composition, alginate encapsulation and microgravity 3D culture: HepG2 cells were able to provide sufficient CYP1A2 but limited CYP3A function.

Intestinal cell lines LS147T and Caco2 and the hepatocyte derived cell line HC-04 were investigated as potential sources to provide CYP3A BAL function. All three were shown to proliferate in BAL specific medium within a 3D culture system. Under these conditions, increased CYP3A function was demonstrated for each cell line, but their usefulness within our BAL was limited.

The effects of increasing HepG2 expression of two nuclear receptors (Constitutive Androstane Receptor and Retinoid X Receptor α) were examined. Within a transient system, increased nuclear receptor expression, in conjunction with chemical induction, resulted in improved function of multiple CYP isoforms; CYP3A function was increased to levels approaching those measured in PHH. Inducible CYP3A function of a stable CAR transfectant was then demonstrated in 3D culture.

Lastly HepG2 cell CYP function was also shown to be source-dependent. Overall it was concluded that through adaptation of culture conditions and genetic manipulation, these cells have the potential to provide CYP function within a BAL.

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List of Abbreviations

[ ] : Concentration

α–MEM: Alpha Minimal Essential Medium

3D: 3-dimensional

6β-OHTST: 6β-Hydroxytestosterone

AhR: Aryl Hydrocarbon Receptor

ACN: Acetonitrile

ADR: Adverse Drug Reaction

ALF: Acute Liver Failure

AMC-BAL: Amsterdam Medical Centre Bioartificial Liver Device

ARNT: Aryl Hydrocarbon Receptor Nuclear Translocator

ATRA: all-*trans* retinoic acid

BAL: Bioartificial Liver Device

bp: base pairs

BCA: Bicinchoninic Acid

BOMFC: Benzyloxymethylfluorescein

BSA: Bovine Serum Albumin

Bsd: Blasticidin S-HCL

CAR: Constitutive Androstane Receptor

C/EBP: CCAAT/enhancer binding protein

CYP: Cytochrome P450

DBA: Dibenz [a,h] anthracene

DBD: DNA Binding Domain

DEPC: Diethylpyrocarbonate

DEX: Dexamethasone

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DR: Direct Repeat

7-EC: 7-Ethoxycoumarin

ECACC: European Collection of Cell Cultures

ECM: Extracellular Matrix

ECOD: Ethoxycoumarin-*O*-Deethylation

EDTA: Ethylene Diamine Tetraacetic Acid

EGF: Epidermal Growth Factor

ELAD: Extracorporeal Liver Assist Device

ER-6: Everted Repeat separated by 6 base pairs

EtOH: Ethanol

FBB: Fluidised Bed Bioreactor

FCS: Foetal Calf Serum

FFP: (Human) Fresh Frozen Plasma

FHH: Foetal Human Hepatocytes

GFP: Green Fluorescent Protein

GI: Gastrointestinal

GST: Glutathione-*S*-transferase

GR: Glucocorticoid Receptor

HBSS: Hanks Balanced Salt Solution

7-HC: 7-Hydroxycoumarin

HGF: Hepatocyte Growth Factor

HLM: Human Liver Microsome

HNF: Hepatocyte Nuclear Factor

HPACC: Health Protection Agency Culture Collections

Hsp90: Heat shock protein 90

IU: International Units

kDa: kilodaltons

LBD: Ligand Binding Domain

LFP: Liver Failure Plasma

3MC: 3-Methylcholanthrene

MDR1: Multiple Drug Resistance 1

MeOH: Methanol

mPMS: 1-Methoxy-5-methyl-phenazinium

NADPH: β-Nicotinamide Adenine Dinucleotide Phosphate

NR: Nuclear Receptor

PB: Phenobarbital

PBREM: Phenobarbital-Responsive Enhancer Module

PBS: Phosphate Buffered Saline

PHH: Primary Human Hepatocytes

PXR: Pregnane X Receptor

RAR: Retinoic acid receptor

RCCS: Rotary Cell Culture System

RE: Response Element

RIF: Rifampicin

RPM: Revolutions per Minute

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction

SOC: Super Optimal Broth for Catabolite Repression

SULT: Sulfotransferase

TAE: Tris-acetate-EDTA

TRH: Thyroid Releasing Hormone

TST: Testosterone

UGT: Uridyl Diphosphate Glucuronosyltransferase

UV: Ultra Violet

VDR: Vitamin D Receptor

VD3: 1α, 25-dihydroxyvitamin D3 (alternatively termed 1,25-$(OH)_2$-D_3)

WST-1: 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt

XRE: Xenobiotic Response Element

XREM: Xenobiotic-Responsive Enhancer Module

Acknowledgements

I would like to thank Dr Clare Selden and Professor Humphrey Hodgson for their supervision and guidance throughout this project. I would also like to thank the Liver Group Charity for funding my studentship and this research.

The HPLC and LC-MS analysis would not have been possible without the advice of Dr Graham Taylor and Dr Nigel Rendell and I am extremely grateful to them for all the help that they gave me.

To all those lab members and other colleagues, past and present, who have provided scientific advice, moral support and a small amount of sanity, thank you.

And finally, the greatest thanks of all go to my family et Ol who have been an unfailing source of patience, wisdom, encouragement and support.

CHAPTER 1 Introduction

Acute liver failure carries a high mortality rate, which could be reduced by the use of a bioartificial liver device (BAL). Within a BAL, a functioning method of xenobiotic metabolism seems essential. The following study investigates Cytochrome P450 (CYP) activity and induction in the human hepatoma-derived cell line HepG2, and the suitability of alternative hepatic and intestinal cell lines for BAL use. CYP activity in human liver was also investigated as a comparator.

In this chapter an overview will be given of:

i) the structure and functions of the liver;
ii) Cytochrome P450 enzymes and their role in drug metabolism;
iii) development of liver support devices; and
iv) *in vitro* methods to measure Cytochrome P450 enzyme function.

1.1 Introduction to the Liver

The liver is the largest internal organ, weighing approximately 1.5kg in an adult human and performs many vital metabolic, synthetic and excretory functions, without which life cannot be sustained [1, 2].

1.1.1 Structure of the Liver

The liver is located in the right upper quadrant of the abdomen. Where it crosses the midline of the trunk it is intersected by the falciform ligament which separates the functionally independent left and right lobes. Each lobe has a dual blood supply and receives oxygenated blood from the aorta via the hepatic artery and a venous supply via the hepatic portal system which drains the spleen, pancreas and gastrointestinal tract. Within the liver, blood vessels drain into sinusoids, which run in channels alongside the acinus (the smallest functional unit within the liver), and this allows liver cells to be perfused with blood. The venous blood is rich in nutrients but also high in toxins from digestive processes and is processed by the liver before being returned to the heart via the hepatic vein. Most waste is removed from the liver by the hepatic bile duct which joins with the cystic duct coming from the gall bladder to form the common bile duct which empties into the duodenum. Other waste is transported in the blood to the kidneys where it is excreted in urine [3].

In order to perform a complex range of functions, the liver is comprised of different classes of cells which are parenchymal and non-parenchymal.

1.1.1.1 Parenchymal Cells

Hepatocytes account for only two thirds of the liver cell population, but fulfil the majority of synthetic, detoxificatory and intermediary metabolic functions. They are large cuboidal cells with central nuclei which may occasionally be tetraploid and binucleate cells also arise. Hepatocytes are highly metabolically active and contain many intracellular organelles including smooth endoplasmic reticulum (lipid/cholesterol synthesis), rough endoplasmic reticulum (protein synthesis), mitochondria (metabolic reactions, Krebs cycle, urea cycle, chemical energy generation), lysosomes, peroxisomes, endocytic vesicles (digestive functions) and storage vacuoles, glycogen granules and fat droplets. Hepatocytes have a unique proliferative capacity which allows the liver to regenerate after injury [4].

Liver parenchyma is homogenous throughout. In liver acini, hepatocytes are functionally polarised with sinusoidal and canilicular poles and form cell plates of about 20 hepatocytes each, which in humans are one cell thick and separated by sinusoids which allow perfusion of blood. Inlet (periportal) and outlet (perivenous) sides of each acinus are exposed to gradients of oxygen, hormones, toxins and metabolites, which form as a consequence of uptake along the sinusoids [5, 6]. This results in a zonated gene expression pattern and metabolic capacity varies along the acinus (Figure 1-1).

Periportal Zone

High O_2 Tension

Hepatocytes contain relatively few but large mitochondria

Plasma Protein Synthesis

• Albumin, Fibrinogen

Metabolism

• Gluconeogenesis

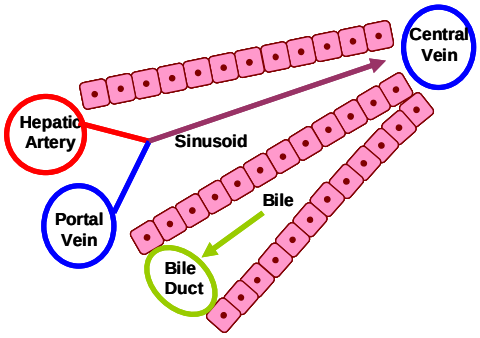
• Glycogenolysis

• Ureagenesis

• Bile acid and cholesterol synthesis

Xenobiotic Metabolism

• Glutathione peroxidation



Perivenous Zone

Low O_2 Tension

Hepatocytes contain more ER

Plasma Protein Synthesis

• α-fetoprotein, α1-antitrypsin

Metabolism

• Glycolysis

• Lipogenesis

Xenobiotic Metabolism

• Mono-oxygenation: Cytochrome P450

• Glutathione conjugation

Figure 1-1. Zonation in the liver acinus. Nutrient rich blood from the portal vein mixes with oxygen rich blood from the hepatic artery in the periportal area and then drains along the sinusoids to the central veins in the perivenous area. Bile flows in the opposite direction into the bile ducts. Hepatocyte function varies along the acinus depending on periportal or perivenous proximity. CYP expression is highest in the perivenous zone where drug metabolism is more extensive.

1.1.1.2 Non-Parenchymal Cells

The main types of non-parenchymal cells in the liver are Kupffer cells, stellate cells and sinusoidal endothelial cells. Kupffer cells are monocyte derived and are resident liver macrophages, responsible for cytokine production and phagocytosis. Kupffer cells are the main source of TNFα production which is involved in stellate cell activation [7]. Quiescent stellate cells are found in normal liver amongst endothelial cells in the perisinusoidal Space of Disse and store droplets of fat and retinoic acid (from Vitamin A) as well as growth factors including hepatocyte growth factor. Following liver damage, stellate cells turn to an activated state which is characterised by a myofibroblast-like phenotype as well as decreased retinoic acid storage. Activated cells provide growth factors and extracellular matrix to promote liver regeneration and are involved in fibrosis [8, 9]. Sinusoidal endothelial cells contain a high amount of endocytic vesicles, are involved in uptake of proteins from the blood and have been implicated in the immune response. These cells are fenestrated to control the passage of particles from the blood through the Space of Disse into the underlying parenchyma [10].

Like hepatocytes, non-parenchymal cells are subject to zonation and are generally more numerous in the periportal region. Increased endothelial fenestrae in the perivenous region allow greater molecular filtering [6].

1.1.1.3 Oval Cells

Oval cells are an inducible progenitor cell population, which are recruited when hepatocytes are unable to regenerate following liver injury; potentially they are activated by stellate cells following ECM production. Oval cells are bipotential and differentiate into hepatocytes or biliary epithelial cells and therefore have the potential to be a major contributing factor to liver regeneration following certain types of injury [11, 12].

1.1.2 Physiological Functions of the Liver

As alluded to above, hepatocytes are responsible for the majority of synthetic, metabolic and detoxificatory functions in the liver. These are reviewed below.

1.1.2.1 Synthetic Function

The liver synthesises many proteins including some for its own processes such as haem and plasma proteins for export including clotting factors, acute phase proteins and most abundantly, albumin which binds toxic molecules, transports hydrophobic molecules through the blood and maintains oncotic pressure [4].

The liver manufactures bile salts used for the emulsification and digestion of lipids and fat soluble vitamins. Bile salts are secreted in bile along canaliculi into the bile duct and stored in the gall bladder and are then released into the small intestine following accumulation of fatty substances. Bile acids can also be used in hepatocytes to excrete fat soluble toxins [4].

1.1.2.2 Metabolic Function

Normal blood glucose levels are maintained by the liver. Excess glucose is converted to glycogen (gluconeogenesis), which is stored in the liver and can be converted to glucose by glycogenolysis. When blood glucose is high, insulin acts to suppress hepatic glucose production, whereas glucagon acts to stimulate glucose release into the bloodstream. When glycogen stores are depleted, fatty acids are released from adipose tissue and converted to ketones to be used as an energy source and eventually muscle is broken down into amino acids to produce glucose [4].

The liver breaks down lipids into their component parts and subsequently secretes cholesterol and triacyglycerol as lipoproteins which can then be distributed around the body in the bloodstream. Cholesterol for example is essential for cell membrane permeability and is a precursor of bile salts and steroid hormones. The liver additionally synthesises cholesterol, although this process is regulated when cholesterol levels are high [4].

Essential amino acids are produced by transamination and modification of dietary amino acids and then reabsorbed into the bloodstream for protein synthesis elsewhere in the body. Excess amino acids cannot be stored and are therefore metabolised by the removal of amino groups (which are then used in gluconeogenesis), resulting in the production of highly toxic ammonia. In the liver, ammonia is converted to urea via the urea cycle which can be excreted by the kidneys. Inadequate detoxification of ammonia occurs in liver failure and results in hyperammonemia; this is thought to be an underlying cause of hepatic encephalopathy [13].

The liver is also responsible for modification of hormones, for example conversion of thyroxin (T4) to the more active tri-iodothyronine (T3) which regulates metabolic functions. Inadequate conversion of T4 to T3 can result in hypothyroidism [14].

1.1.2.3 Biotransformation and Excretion

Biotransformation of xenobiotics in the liver is a multistep process which is carried out by hepatocytes.

Phase I metabolism reactions include oxidation, reduction and hydrolysis of the substrate to add (or unmask) a functional group. For drug molecules, Phase I reactions are mainly represented by oxidations by Cytochrome P450 monooxygenases which will be described in further detail in section 1.2.

Flavin-containing monooxygenases (FMOs) are another group of microsomal monooygenase enzymes that contribute to hepatic Phase I metabolism. These enzymes are polymorphically expressed in humans but unlike CYPs are less readily inhibited or inducible. The number of characterised FMOs is relatively small compared to CYPs: there are currently 5 identified functional human FMO enzymes, FMO3 is the most

predominant in human hepatic metabolism. Compared to CYP enzymes, the role of FMOs in xenobiotic metabolism is less well documented (although FMOs exhibit a higher degree of thermal instability *in vitro*, which may cause a metabolic shift to CYP activity). With respect to drug metabolism, there is a degree of substrate overlap between CYPs and FMOs, but it is generally accepted that CYP enzymes have the more significant role in xenobiotic metabolism [15, 16].

Other enzymes involved in oxidative metabolism include molybdenum oxygenases, prostaglandin H synthase, lipoxygenases, amine oxidases, and alcohol and amine dehydrogenases [17].

Phase II conjugations modify the functional group added in Phase I (although conjugations can occur without Phase I metabolism for some substrates) to improve polarity therefore increasing excretion into the bile or urine. The most common Phase II reactions are glucuronidation, involving coupling with glucuronic acid via UDP-glucuronosyltransferases (UGT), and sulphation, involving coupling with sulphate via sulphotransferases (SULTs). Other important Phase II enzymes include glutathione-S-transferase (GST), arylamine *N*-acetyltransferase (NAT) and epoxide hydroylase (EPHX) [18, 19].

Phase III elimination prevents intracellular accumulation of Phase II conjugation products. This involves removal of metabolites to the extracellular medium by active transport using membrane transporters including P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and organic anion transporting polypeptide 2 (OATP2).

The biotransformation processes described above facilitate the detoxification and excretion of many xenobiotics, but in some instances can promote mutagen formation. For example benzo [a] pyrene, which is found in cigarette smoke and chargrilled meat, is converted to a carcinogenic metabolite by CYP1A and EPHX [18, 20].

1.2 Cytochrome P450 Enzymes

Cytochrome P450 (CYP) enzymes are the most prominent group of drug metabolising enzymes in humans, responsible for as much as 90% of xenobiotic metabolism. Their name derives from their structure (they contain a cytochrome b-type haemprotein) and the discovery that they absorb light at 450nm when the reduced form of the protein is bound to carbon monoxide (Figure 1-2). CYP enzymes are haemproteins and are comprised of a heme group (Protoporphyrin IX) and an apoprotein of 44-60 kDa. The porphyrin is common to all CYP enzymes (and other hemeproteins), however the apoprotein varies between different CYP isoforms and this accounts for the differences in their properties, including molecular weight, substrate and product specificities [21].

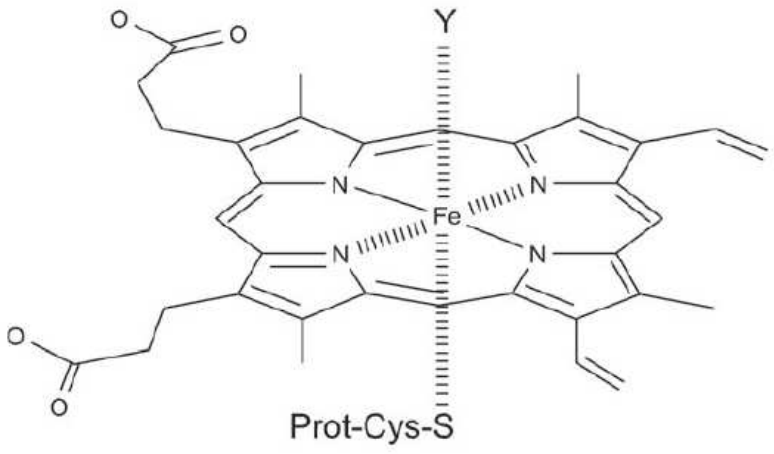


Figure 1-2. Cytochrome P450 enzymes contain haem iron bound within a porphyrin ring (Protoporphyrin IX). Four ligands to the haem iron are provided by the nitrogens on the heme ring. Above and below the plane of the haem iron there is room for two additional (non-porphyrin) ligands. In Cytochrome P450 proteins the 5th ligand (Prot-Cys-S) is a thiolate anion which comes from a cysteine at the haem binding region of the active site. The 6th postion (Y) is available to bind small molecules such as carbon monoxide which have a high affininty for the ferrous haem. Image obtained from [21].

CYP enzymes are expressed in many tissues throughout the body, including the gut wall, kidney, lung, placenta, skin (not 3A4, 1A2 [22]), brain and blood cells [23, 24], but they are located at highest concentrations within the liver. The xenobiotic metabolising forms of CYP enzymes are membrane bound within the smooth endoplasmic reticulum (ER). CYP enzymes are anchored to the membrane bilayer of the ER by their N terminal segments and the remainder of the protein is exposed to the cytosolic compartment allowing access to the active site of the enzyme. The cofactor NADPH

cytochrome P450 reductase, which donates electrons for CYP catalysis via electron transfer chain, is similarly located and thus is associated with CYP enzymes in the ER.

The mechanism of CYP catalysis has been extensively characterised and described [21, 25-27]. CYP mediated catalysis consists of a two stage reaction, using electrons provided from NADPH via associated NADPH CYP reductase for the reduction of molecular oxygen and subsequent single oxygen insertion into substrate molecules reversibly bound to the active domain of the CYP enzyme. A simplified summary of this is shown in Figure 1-3.

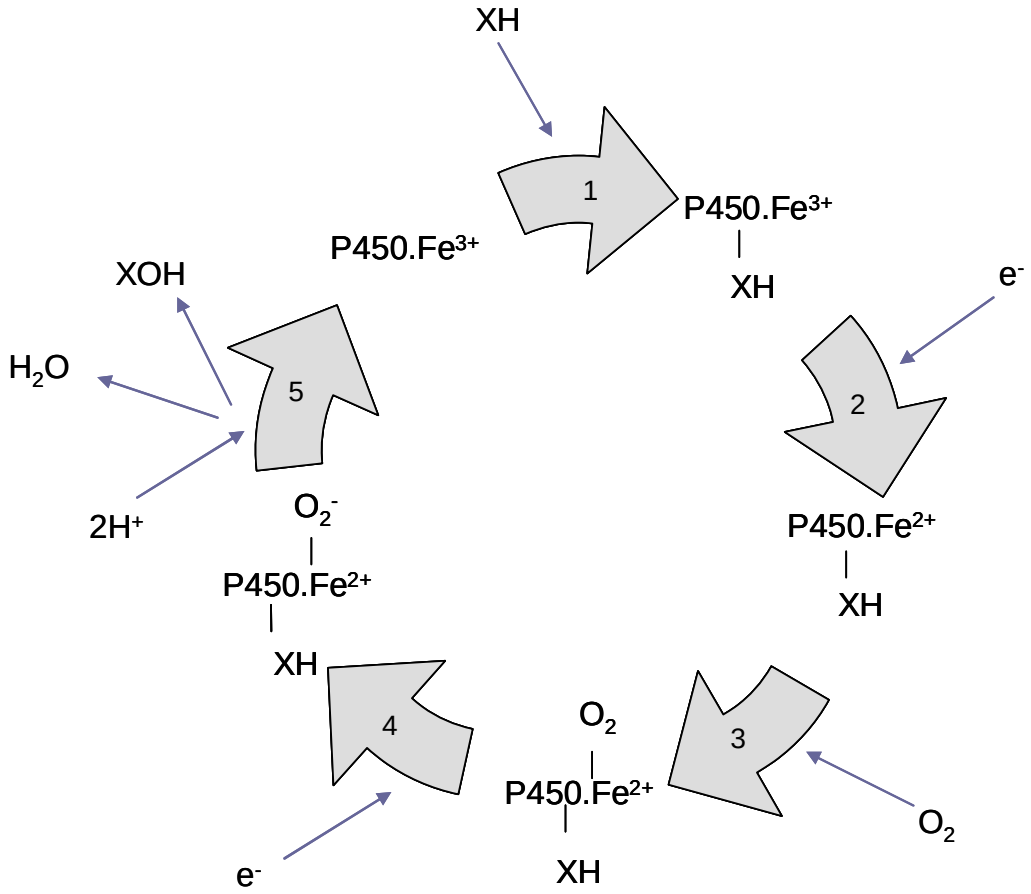


Figure 1-3. Cytochrome P450 catalysis. Upon binding of the substrate (X) to the CYP enzyme (1), the spin state of the heme bound iron changes from resting to active allowing it to accept electrons from NADPH via NADPH cytochrome P450 reductase (2). This changes the iron oxidation state from Fe^{3+} to Fe^{2+} resulting in binding of molecular oxygen (3), which is then reduced and cleaved (4). CYP enzymes are monooxygenases, and so incorporate one atom of an oxygen molecule the substrate, whilst the other is reduced to water (5).

1.2.1 Cytochrome P450 Nomenclature

A universally recognised nomenclature system for CYP enzymes was devised in 1987 and there are now more than 9000 named Cytochrome P450 sequences. All new CYP genes that are discovered are submitted to the Committee on Standardized Cytochrome P450 Nomenclature for naming [28]. Cytochrome P450 proteins are categorised into families and subfamilies by their sequence similarities (Table 1-1). Each isoform is prefixed by CYP. Sequences that are greater than 40% identical at the amino acid level belong to the same family denoted by a number. Sequences demonstrating greater than 55% similarity belong to the same subfamily as denoted by a letter, with a final number representing each individual enzyme.

Table 1-1. Cytochrome P450 Nomenclature.

Category	Addition to CYP	Example
Family *> 40% amino acid identity*	**Number**	CYP**1**
Subfamily *>55% amino acid identity*	**Number** **+ Letter**	CYP**1A**
Single Gene/Protein	**Number** **+ Letter** **+ Number**	CYP**1A2**

1.2.2 Human Cytochrome P450 in Xenobiotic Metabolism

To date, there are 18 known families of human Cytochrome P450, of which 3 play a major role in xenobiotic metabolism: CYP1, CYP2 and CYP3A (Figure 1-4).
Other CYP families play important roles, including the metabolism of endogenous compounds and cholesterol, steroid and lipid biosynthesis, however, this thesis will focus upon CYP1A and CYP3A since the former is readily inducible in BAL relevant cell lines, and the latter is deemed necessary for a BAL since it demonstrates the least substrate specificity, is the most abundantly expressed CYP subfamily in adult human liver and plays the greatest role in drug metabolism.

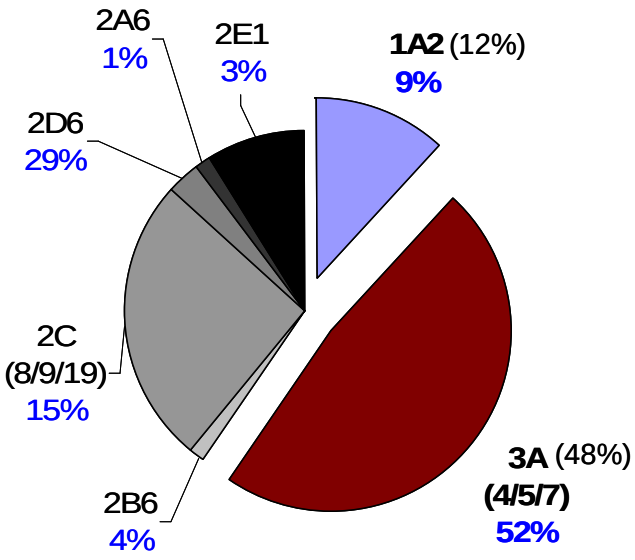


Figure 1-4. Contribution of key Cytochrome P450 isoforms to xenobiotic metabolism in humans. The pie chart indicates % hepatic expression and values are shown for CYP1A2 and CYP3A. The blue values below correspond to the % of drugs metabolised by each CYP. Data was adapted from[27, 29-31].

1.2.3 Species Specificity of CYP Expression

Hepatic expression of CYP1A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A subfamilies arises in other mammalian species including rat, mouse, dog, rabbit, pig and primates, although expression of individual CYP isoforms is species specific. The predominant isoform within the same CYP family is usually different among animal species [32, 33]. An exception to this is CYP1A which is present across species, with CYP1A2 as the predominant hepatic isoform. With regard to CYP3A, in adult humans CYP3A4 is generally predominant, but this varies for other species including rat (CYP3A1/23), dog (CYP3A1/2), monkey (CYP3A8), rabbit (CYP3A6) and pig (CYP3A1/2) [30, 34]. These species are all reported to metabolise common CYP3A substrates including nifedipine, midazolam and testosterone [35, 36], although there are some species differences in metabolite profiles. For example the main metabolite of testosterone is 6β-hydroxytestosterone (6β-OHTST) in human hepatocytes, but not rat (17-OHTST), rabbit (16α-OHTST) or dog cells (17-OHTST); pig hepatocytes have a more similar testosterone metabolite profile to humans [29].

In contrast to substrate metabolism, there are marked differences in species responses to inducers of both CYP1A and CYP3A. Omeprazole is a potent inducer of CYP1A in human, dog and pig hepatocytes but not in rat or mouse. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is reported to preferentially induce CYP1A1 in rat hepatocytes and CYP1A2 in human hepatocytes [37]. With regard to CYP3A, rifampicin is a potent inducer of CYP3A in humans, rabbits, pigs and dogs but is ineffective in rats. Dexamethasone is a less potent inducer of CYP3A than rifampicin in humans, and is ineffective in pigs and dogs, but strongly induces this subfamily in rats [35, 38]. One proposed reason for species-specific mechanisms of CYP induction is structural differences in the ligand binding domain of nuclear receptors which mediate CYP induction [37, 39]. The molecular mechanisms of CYP induction will be addressed further in section.1.2.6.

1.2.4 Specific Characteristics of CYP Isoforms

The mechanism of CYP dependent catalysis is thought to be consistent across isoforms and it is therefore the relationship between active site and substrate which governs the isoform responsible for metabolism [40, 41]. For each isoform, enzyme-substrate interaction may be governed by factors including (i) topography of the active site, (ii) the extent of steric hindrance of the CYP iron-oxygen complex to possible sites of metabolism and (iii) binding affinity of the substrate and subsequent hydrogen/electron extraction.

CYP enzymes show an affinity for a diverse range of substrates and individual isoforms exhibit distinct but overlapping substrate specificities; only a few xenobiotics are metabolised exclusively by one enzyme. For human CYP enzymes, some general relationships have been identified between the physicochemical properties of ligands and their interactions with the major CYP isoforms including CYP1A2 and CYP3A4.

1.2.4.1 Cytochrome P450 1A2 Structure Activity Relationship

The active site of CYP1A2 is modelled as a rectangular cavity whose size and shape is restricted by aromatic side chains. Substrates undertake $\pi - \pi$ interactions with the aromatic portions of the active site, and hydrogen bonding further from the active site. CYP1A2 substrates have been characterised as neutral, basic or lipophilic planar polyaromatic or heterocyclic amines and amides with a large area/depth ratio and at least one putative H-bond donating site. Examples of CYP1A2 substrates include

acetanilide, aromatic amines, caffeine, lidocaine, paracetamol, phenacetin and R-warfarin. CYP1A2 metabolism is associated with the metabolic activation of procarcinogens partly due to the formation of reactive intermediates [21, 42].

1.2.4.2 The CYP3A Subfamily

In terms of hepatic expression and amount of drugs metabolised, the CYP3A subfamily is predominant in adult human liver. Currently there are four identified CYP3A genes in humans: CYP3A4, CYP3A5, CYP3A7 and CYP3A43. CYP3A4 is the major isoform expressed in adult human liver but expression in adults is varies up to 100-fold [43-45]. CYP3A7 is the major CYP3A in foetal liver and expression decreases after birth, although has been detected at low levels in many human adults (it contributes around 2% of total CYP3A although this value may increase up to 36% in some individuals [46]). CYP3A43 contributes the least to adult hepatic CYP3A (0.1% of CYP3A4 expression) and its reported role in CYP3A mediated metabolism is negligible [47]. CYP3A5 is polymorphically expressed, although where present (about a third of adult livers) can comprise up to 50% of hepatic CYP3A [48].

The majority of CYP3A substrates can be metabolised by all CYP3A isoforms, with CYP3A4 having the highest metabolic capacity, although there are exceptions. Cisapride is not well metabolised by CYP3A7 [48-50]. CYP3A is the major subfamily involved in testosterone metabolism, but CYP3A7 does not substantially contribute to testosterone 6β-hydroxylation [51, 52]. CYP3A7 has a greater catalytic activity towards 16-α hydroxylation of dehydroepiandrosterone (involved in estriol biosynthesis [53]) than CYP3A4.

CYP3A induction is also differentially upregulated in humans. CYP3A4 expression exclusively is upregulated by St Johns Wort. Rifampicin exposure leads to induction of CYP3A4 and to a lesser extent CYP3A7 but has minimal effect on CYP3A5 [54-56]. Dexamethasone, although less potent than rifampicin, induces the expression of (in order of potency) CYP3A4, CYP3A7 and CYP3A5 [57].

1.2.4.2.1 Cytochrome P450 3A4 Structure Activity Relationship

CYP3A4 is the most widely expressed CYP in adult human liver and demonstrates the broadest substrate specificity. It is an allosteric protein and exhibits atypical kinetics for which several models have been proposed; these centre around either multiple substrate binding at a single active site or multiple substrate binding at distinct active sites within the enzyme [58-60]. As a result of this, conflicting events can occur during substrate metabolism such as enzyme activation and inhibition.

Some common features related to CYP3A4 binding are hydrogen bonding and $\pi - \pi$ stacking, but substrates are structurally diverse. Examples of CYP3A4 substrates include caffeine, cyclosporin, HIV protease inhibitors, androstenedione, lidocaine, midazolam, omeprazole and testosterone [21].

1.2.5 Factors Influencing CYP Function

Poor pharmacokinetics is a major contributing factor to adverse drug reactions (ADR) as well as to the attrition of compounds during the drug discovery and development process, therefore variations in Cytochrome P450 function can have considerable impact in a clinical setting.

In humans, there is considerable variation in CYP expression and this may be attributed to several factors.

Genetic polymorphisms in CYP expression can result in low metabolic capacity and have been characterised for a number of isoforms. For certain populations, polymorphisms have a particular impact on CYP2C19 and CYP2D6 activity. Up to 20% of the Asian population are carriers of null CYP2C19 alleles resulting in poor CYP2C19 metabolism and 5-10% of Caucasians and 0-1% of Africans and Asians are poor CYP2D6 metabolisers [31].

As mentioned above (section 1.2.4.2), polymorphisms related to CYP3A5 and CYP3A7 expression in adult liver have also been identified [43, 46]. Although genotypic variations

in CYP1A2 and CYP3A4 genes have been described, functional variations in these isoforms are rarely attributed to genetics [30, 31].

CYP variation may arise due to environmental factors, such as exposure to alcohol and tobacco, pollution and diet. For example, smokers present with higher expression of CYP1A and exposure to environmental pollutants such as polycyclic aromatic hyrdrocarbons (PAH) has a similar effect [24, 61]. Acetaminophen toxicity is enhanced by alcohol consumption due to the inductory effect of ethanol on CYP2E1, [62] and dietary components including chargrilled meat, cruciferous vegetables and green tea all cause induction of CYP1A [63]. Certain disease pathologies, notably hepatic cirrhosis and steatosis are associated with decreased CYP function, and down-regulation of CYP enzymes arises in humans during inflammatory response in the liver [64-68].

Most human CYP enzymes do not show marked sexual differentiation in expression and there is a large variation in CYP activity in both males and females. CYP1A2 activity has been shown to be higher in human liver microsomes isolated from males although not all clinical studies support these findings. Liver microsomal CYP3A4 activity was also shown to be higher in females in the same study, and some, but not all, clinical studies support this finding [68]. There are many factors that may contribute to apparent sex differences in drug metabolism including the use of oral contraceptive steroids in females, therefore meaningful differences in the human population are difficult to ascertain. Similarly, age-related differences in CYP function in humans are difficult to interpret.

Most commonly, ADR arise as a result of polypharmacy causing drug-drug interactions, resulting in pharmacological potentiation (apparent overdosing) or decreased clinical response (underdosing). Both of these effects are caused by CYP enzymes.

1.2.5.1 Cytochrome P450 Inhibition

Pharmacological potentiation arises as a result of Cytochrome P450 inhibition. This response occurs almost immediately post exposure and can occur through competitive inhibition (substrate and inhibitor binding the same active site), mechanism based inhibition (inactivation of CYP by a drug metabolite), or may be non-specific (caused by inhibitor binding directly to the heme portion of CYP) [29].

1.2.5.2 Cytochrome P450 Induction

Clinically, induction of CYP isoforms can lead to a decrease in the therapeutic efficacy of medications (a well documented example is induction in the metabolism of the CYP3A4 substrate cyclosporin by rifampicin resulting in organ graft rejection), or create an imbalance between detoxification and activation by the increased production of reactive metabolites, particularly in the case of CYP2E1 [69].

In contrast to CYP inhibition, induction is a slow regulatory process which commonly occurs by increased gene transcription and protein synthesis. However alternative mechanisms of CYP induction involve post translational stabilisation of the CYP enzyme or inhibition of the enzyme degradation pathway. For instance ethanol mediated CYP2E1 induction [70, 71] and CYP3A induction in rats following administration of troleandomycin arise through protein stabilisation [37].

Compounds causing *de novo* synthesis of CYP enzymes act through receptor mediated mechanisms involving nuclear receptors.

1.2.6 CYP Induction by Nuclear Receptors

Nuclear receptors (NR) comprise a family of ligand activated transcription factors and are responsible for the xenobiotic mediated induction of a number of genes involved in Phase I (CYP enzymes), Phase II (GSTs, SULTs) and Phase III metabolism (drug transporters) [72-74]. Sequencing of the human genome resulted in the identification of 48 NRs, of which half are classified as orphan receptors since they have no known natural ligands. Within this category are true orphans (no known ligand) or adopted orphans (candidate ligands recently identified) [75].

A unified nomenclature system for the NR super-family was adopted in 1999 (Nuclear Receptor Nomenclature Committee 1999). This phylogeny based system divides NR into subfamilies, groups and genes based upon their sequence alignment: generally members of the same group share at least 80-90% identity in the DNA binding domain and at least 40-60% in the ligand binding domain [75, 76]. The system was based on the CYP nomenclature system and provides a logical numbering system and receptor code to support the trivial names given to many NR. For example RXRα is alternatively termed NR2B1 [75].

CYP inducers bind to, or activate, the aryl hydrocarbon receptor (AhR) or nuclear receptors (NR) including pregnane X receptor (PXR) and constitutive androstane receptor (CAR). AhR and CAR are located predominantly in the cytoplasm and translocate to the nucleus following activation. PXR has been detected in the cytoplasm but is widely thought to be concentrated in the nucleus [37].

Once activated, NR form heterodimers with a co-receptor which then binds to response elements located in the promoter region of the CYP gene, leading to increased transcription (Figure 1-5).

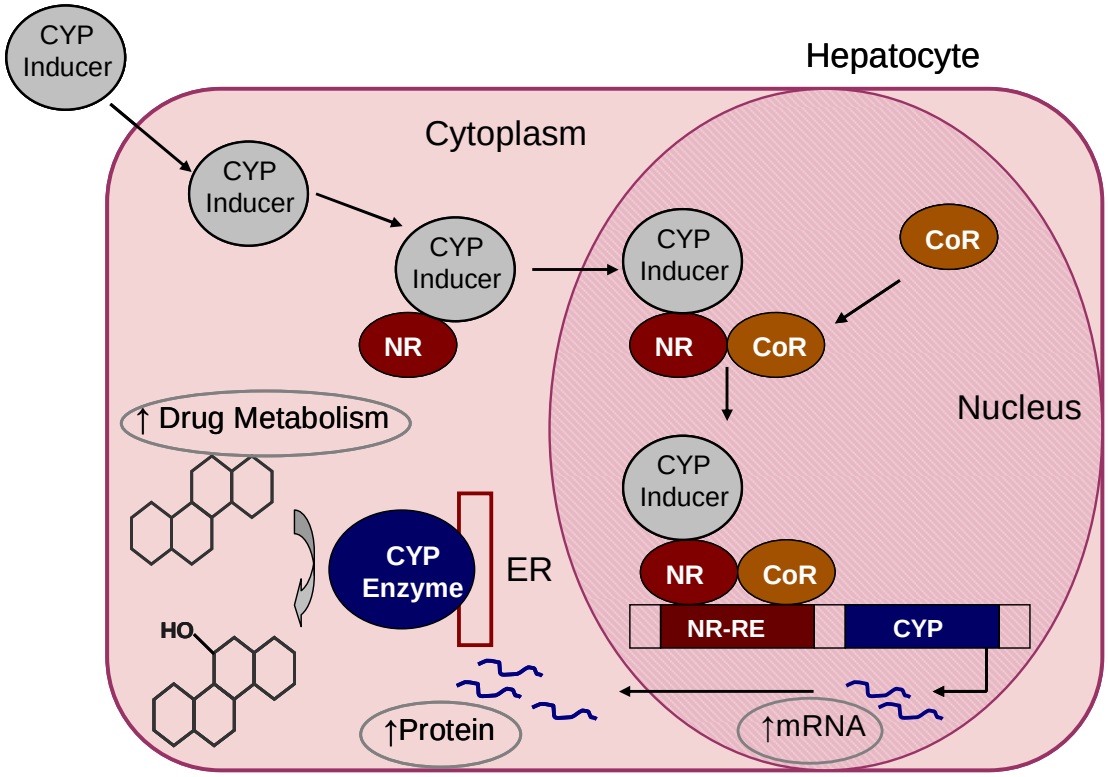


Figure 1-5. Mechanism of CYP induction: Nuclear Receptors (NR) form heterodimers with a co-receptor (CoR) in the presence of CYP inducers. Heterodimer binding to Response Elements (NR-RE), located in both proximal and distal CYP gene promoters, leads to increased transcription of CYP mRNA and increased xenobiotic metabolism. Adapted from[77].

1.2.6.1 Aryl Hydrocarbon Receptor and CYP1A Induction

The main chemical class responsible for CYP1A induction is aromatic hydrocarbons [78], and it is activation of the aryl hydrocarbon receptor (AhR) which results in increased expression of CYP1A mRNA. Unliganded AhR is retained in the cytoplasm as a

stabilised protein complex also comprising two molecules of hsp90, XAP2 and p23. Following agonist binding, AhR undergoes a transformational change and is translocated to the nucleus where it loses its chaperone partners and forms a heterodimer with aryl hydrocarbon nuclear translocator (Arnt). AhR and Arnt both belong to the basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) protein family and it is these domains which drive heterodimer formation and DNA binding. AhR-Arnt heterodimers bind xenobiotic response element (XRE) in the proximal and distal promoter regions of the CYP1A gene resulting in transcription[79].

The greatest exogenous source of AhR ligands are dietary components, including vegetables and teas, and environmental pollutants. In addition to CYP1A1, CYP1A2 and CYP1B1, AhR mediated induction also regulates the expression of Phase II enzymes (UGT1A1, SULT 1A1, GST-A2) and multi-drug transporters (MDR1) [30, 79, 80].

1.2.6.2 Nuclear Receptor Mediated CYP3A induction

As is the case with its substrates, CYP3A inducers may also be broad ranging. Ligands that bind the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) both cause induction of CYP3A following heterodimerisation with RXRα.

PXR (NR1I2) and CAR (NR1I3) are members of the same NR subfamily and have closely related sequences: in humans they share 66% sequence identity in the DNA binding domain and 45% in the ligand binding domain [81]. PXR and CAR also share the same co-receptor (RXRα) and can signal through the same response elements.

1.2.6.2.1 Pregnane X Receptor

Although its primary target gene is CYP3A4, PXR also mediates the induction of CYP3A7, CYP2B6, CYP2C9 and CYP2C8, as well as UGTs, GST, and MDR1 and OATP2. PXR is activated by a wide variety of structurally diverse chemicals including drugs such as rifampicin, steroid hormones including progesterone, and dietary components such as St. John's wort [77, 82].

PXR/RXRα heterodimers bind specific response elements in the CYP3A gene. These elements contain two nuclear receptor half-sites of the consensus sequence AG(G/T)TCA arranged as different configurations such as DR3 (direct repeat with 3 nucleotide spacing) or ER-6 (everted repeat separated by 6 base pairs).
In the proximal promoter of CYP3A4, the response element is an ER6 motif.
The distal xenobiotic responsive enhancer module (XREM) contains three elements dNR1 (DR-3), dNR2 (ER-6) and dNR3 (DR3). Transfection experiments have shown that binding at both elements is required for maximum PXR transactivation. Further upstream from the distal element is a far enhancer containing an ER-6 element and binding here, along with the other response elements, further synergistically increases CYP3A4 transactivation [83].

The XREM is conserved in CYP3A7 and the motifs within can mediate PXR and CAR dependent induction. Like CYP3A4, the CYP3A7 promoter contains an ER6 motif which is responsive to rifampicin, however it contains two mutations which decrease affinity for PXR and CAR [56, 84]. High CYP3A7 expression in adult liver is associated with an allele that contains the CYP3A4 ER-6 motif [46, 84]. Other mutations in the CYP3A7 ER-6 promoter may increase CAR but not PXR mediated transcription [84].

Splice variants of PXR have been identified in human liver; PXR2 and PXR3 represent ~7% and 0.3% of total PXR transcripts and lack a portion of the LBD [85, 86]. It is proposed that PXR1 and PXR2 have comparable transcriptional activity for CYP3A4 but the splice variant has a restricted range of activators [87].

1.2.6.2.2 Constitutive Androstane Receptor

CAR was first identified as a mediator of CYP2B6 induction which heterodimerises with RXRα and then binds DR4 motifs in the phenobarbital-responsive enhancer module (PBREM) of the CYP2B6 promoter. CAR is now recognised as a mediator of CYP3A induction as well as CYP2C, CYP2A, SULT1A1, OATP2 and MRP3 [30, 80].

CAR is transcriptionally active in the absence of ligand, and its activity is repressed upon binding of steroid ligands (androstanol and androstenol), which act as inverse agonists and prevent transactivation. Upon exposure to inducer, CAR translocates to the nucleus and activates its target genes. CAR localisation is regulated by dephosphorylation and translocation can be inhibited by phosphatase inhibitors [37, 82].

CAR is also activated by structurally diverse compounds, however it has a more restricted ligand profile than PXR. This can partly be explained by modelling studies which have demonstrated that the LBD of PXR is more accommodating to a wider range of compounds [81]. PXR and CAR share many ligands, however there are transcriptional differences. For example, androstenol and clotrimazole are both activators of PXR that inhibit CAR [82]. In the case of CAR, ligand binding alone may be insufficient to induce CAR translocation into the nucleus [74]. Phenobarbital is an indirect inducer of CAR which does not bind the receptor yet causes translocation.

As described, PXR and CAR share response elements, hence both can activate multiple CYP genes (cross-talk). In the CYP2B6 gene, binding of CAR/RXRα predominates at the PBREM promoter, although this can also be bound by PXR/RXRα

Like PXR, CAR binds the ER-6 motif in the CYP3A4 proximal promoter and the dNR1 portion of the XREM; co-operation is required between the two response elements for CYP3A4 activation. Within the XREM, CAR has a weaker affinity than PXR to the dNR2 and dNR3 regions [88].

The CAR gene also undergoes alternative splicing. Effects of these splice variations in human liver are reported to include increased and decreased ligand dependent activity, and decreased formation of RXRα heterodimers [89-92].

1.2.6.3 Nuclear Receptor Cross-Talk

The signalling pathways of CAR and PXR converge at common response elements, for example in the CYP3A4 gene, and therefore they regulate overlapping sets of target genes. Moreover RXRα is a promiscuous nuclear partner forming heterodimers with multiple NRs and overlapping induction pathways are not limited to CAR and PXR. For example, upon activation, the vitamin D receptor (VDR), forms an RXRα heterodimer and induces the expression of CYP3A4, 2B6 and 2C9 (through ER6, DR3 and DR4), therefore competing for CYP regulation [93, 94].

For CAR, PXR (and VDR) mediated induction, RXRα is an obligate dimerisation partner in CYP induction, therefore decreased RXRα leads to diminished NR mediated responses and vice versa.

CAR, PXR and RXRα expression have all been demonstrated to be under control of the glucocorticoid receptor (GR) and glucocorticoids (including dexamethasone and hydrocortisone) increase expression of these receptor proteins. Pascussi *et al* [95-97] propose a two step model whereby physiological concentrations of glucocorticoids act at GR to increase CAR, PXR and RXRα levels, and high concentrations of glucocorticoids directly activate PXR and CAR. GR activation may also have a direct role in CYP expression since glucocorticoid response elements have been identified in the CYP3A5 and CYP2C9 gene promoters.

An alternative mechanism for NR cross-talk is biotransformation. For example the AhR antagonist omeprazole sulphide is metabolised by CYP3A4 to the AhR agonist omeprazole which in turn results in increased CYP 1A expression. This process is therefore increased in the presence of CYP3A4 induction [73].

1.3 Liver Disease and Treatment

Severe liver injury can result from toxic insult such as acetaminophen overdose or mushroom poisoning. Damage can also occur after exposure to other injurious agents, for example viruses and alcohol, which can cause massive and acute hepatic damage, or over a longer period of time, they may cause chronic liver injury leading to eventual fibrosis and then cirrhosis culminating in liver failure [98]. Metabolic diseases causing liver failure include Wilson's disease in which an accumulation of copper causes liver damage [99]. Liver failure may also arise as an idiosyncratic drug reaction, as a complication of pregnancy or may (rarely) be idiopathic.

1.3.1 Acute Liver Failure

Acute liver failure (ALF) has a 60-90% mortality rate depending on the underlying cause [100]. Early symptoms include altered mental status, jaundice and abnormal blood clotting factors progressing to hepatic encephalopathy and multi-organ failure [98]. In addition to general therapies to treat these symptoms (such as administration of Vitamin K to treat coagulopathy), there are also some specific treatments available for certain etiologies (N-acetylcysteine (NAC) is an effective antidote for acetaminophen hepatotoxicity, Penicillin G and silibinin (silymarin or milkthistle) are antidotes for mushroom poisoning) [215]. A consideration relevant to the treatment of ALF is, that if metabolised by the liver (in particular by Cytochrome P450 enzymes), drugs will have a longer half-life due to delayed clearance. In these instances, drug exposure can be limited by administering smaller doses. All non-essential medications can also be withdrawn.

Although individual symptoms of ALF can be mitigated by medical management, no agent or therapy that is beneficial to all patients has been identified. Whilst there are some patients who are able to achieve regeneration of sufficient liver mass to sustain survival, the only effective treatment for acute liver failure, currently recognised, is orthotopic liver transplantation. However, there is a shortage of donor organs, progression of disease is often too quick for a suitable donor organ to be found and contraindications to transplant may exist. Though a liver assist device could provide liver function in order to correct the manifestations of ALF, decades of research have thus far failed to identify a reliable liver support system with clear therapeutic benefit.

1.4 Extracorporeal Liver Support as a Treatment of ALF

Extracorporeal liver support devices could provide short term support to liver function in patients with ALF and would serve as a bridge to liver transplantation or to liver regeneration. Providing time for liver regeneration would reduce demand for donor organs and also eliminate the necessity for the lifelong immunosuppressant regimen that arises as a consequence of transplantation.

Existing devices can be divided into two categories: artificial (detoxificatory) or bioartificial liver devices.

1.4.1 Artificial Liver Devices

Artificial liver devices were designed to remove toxins from patient plasma that cause hepatic encephalopathy and multi-organ failure and work using one or more of the following methods:

i) membrane filtration to remove large molecules;
ii) dialysis of smaller molecules by diffusion exchange; and/or
iii) molecular adsorption by charcoal, resins or albumin.

Devices that have undergone clinical evaluation include single pass albumin dialysis (SPAD), Molecular Adsorbents Recirculating System (MARS) and the Prometheus (fractionated plasma separation and adsorption) system.

SPAD is based on albumin dialysis whereby an albumin dialysate acts as an acceptor for toxins. MARS is similar to SPAD, but includes an additional step to partially cleanse the albumin for recirculation by charcoal adsorption and anion exchange. In the Prometheus system, filtered plasma is passed through two adsorption columns (a natural resin and an anion exchange resin) to remove albumin bound toxins and this is combined with hemodialysis to remove water soluble toxins [98, 101-105].

These devices have had mixed success clinically. They have significant impact on removal of plasma toxins, but do not improve survival in ALF patients. The limitations of these devices probably reflect their non-specificity when removing compounds and their inability to replace the synthetic capacity that is lost when liver function is

compromised. Attempts to address these shortfalls have been made through the design of bioartificial liver devices.

1.4.2 Bioartificial Liver Devices

Bioartificial liver devices (BAL) contain a biological (cellular) component which performs liver specific functions such as protein synthesis, ammonia detoxification and drug metabolism. A number of BALs have undergone clinical evaluation, however, proof of success in a controlled human trial is lacking [98].

In order to successfully develop a BAL which, in addition to performing liver specific functions, is widely and readily available, certain biological and technical aspects should be addressed.

i) Cell type and source

Choice of cell type is dependent on both function and availability. Hepatocytes are responsible for the majority of liver functions (see section 1.1.1), and so form the key component of a BAL. Although human hepatocytes may appear the strongest candidate, their use is restricted by a number of factors. Other cell types that have been considered in BAL systems include xenogeneic hepatocytes, cell lines of human liver origin, foetal hepatocytes and, most recently, stem cells. Advantages and limitations of potential cell types will be discussed in section 1.5.

ii) Cell number required to replace liver function

The hepatocyte number in an adult human liver is in the order of $2x10^{11}$ (assuming a 1.5kg liver). Within a BAL, the provided cell function will be additive to the residual function of the patient's damaged liver and restoring as little as 10% of liver function could make a critical impact. A biomass equating to this amount of normal liver cell mass (150g of cells) has been proposed by others as minimum required for a BAL to provide liver function [106]. This assumes, however, that function of supporting cells equals hepatocytes *in vivo*. Taking a more cautious approach, the Liver Group (LG) BAL provides for a cell number of $7x10^{10}$ cells or 30% of hepatocyte cell mass.

iii) Culture format

Classical monolayer cultures are not appropriate for BAL, not least because an extremely large surface area would be needed to achieve a sufficient cell number. Dead

space and culture volume can be minimised in bioreactor culture and this is achieved by cell aggregation into a 3D culture format either by encapsulation or mechanical promotion of spheroid formation, or by allowing cells to proliferate and populate a defined space (hollow fibre). Maintaining hepatocytes in 3D culture also has beneficial effects on differentiation.

In monolayer culture, hepatocytes flatten and spread following attachment. This results in loss of polarity and cuboidal shape, which disrupts their natural cell architecture. Improved differentiated function can be achieved *in vitro* by culture on extracellular matrix (ECM) such as collagen, but use of ECM is difficult in a bioreactor culture that allows direct contact with patient plasma - collagen promotes clotting factors and soluble ECM components could be released into the patient's circulation. Additionally the majority of ECM preparations are animal derived, with therefore some biological hazard.

In hepatocytes, perturbation of microtubule network has a deleterious effect on cell functions including NR signalling [107, 108], hence CYP expression. The use of 3D culture systems promotes cell organisation to resemble that found *in vivo*, the cytoskeletal network and polarity of hepatocytes is maintained and this results in improved differentiated function including xenobiotic metabolism [109-112].

iv) Bioreactor design

In terms of bioreactor architecture, cells may be anchored to a matrix in 3D configuration with interwoven fibres providing gas exchange or plasma perfusion (hollow fibre devices). Alternatively encapsulated hepatocyte systems may be used, in which cell performance can be improved by a microgravity environment that provides decreased sheer stress and increased mass transfer. Cells in the device may be perfused by either whole blood or plasma which may come into direct cell contact or be separated by a mass transfer membrane [98, 103, 105, 113]. Environmental factors including supply of gases, flow rate and pH as well as nutrient replenishment can be tightly controlled, so that a physiological environment can be maintained during mass cell culture. Cryopreservation and transport of bioreactor cultures are also important considerations.

As described above, there are numerous constraining factors in BAL development. Early devices were designed using cells with limited function; these have allowed improvements in bioreactor design and culture conditions in order to generate and maintain larger biomasses. As the next step, identification of a more suitable cell source seems imperative. This involves the identification of cells that are obtainable on a large scale which are able to perform a full range of liver functions over a prolonged period of time.

1.5 Sources of Cells That May be Considered for a BAL in Order to Establish a Functioning Cytochrome P450 System

As the major drug metabolising enzyme in humans, achieving a functioning Cytochrome P450 system in bioartificial liver is critical. Potential cell sources which could provide this function will be discussed below.

1.5.1 Primary Human Hepatocytes

Primary human hepatocytes (PHH) have been extensively studied as *in vitro* models of liver function and are able to fulfil, to some extent, each of the metabolic demands required of BAL. However the use of PHH is impractical for a number of reasons. Availability is limited: liver cell mass obtained from partial hepatectomy is insufficient, explanted organs are preferentially used for transplantation and in discarded donor organs hepatocyte quality is generally compromised by steatosis and/or cirrhosis or traumatic insult. Bioreactor cultures comprised of PHH obtained from individual donor organs require extensive characterisation [113]. This is of particular relevance to xenobiotic metabolism due to the large range of interindividual variability in CYP expression levels that arises between donors (as discussed in section 1.2.5 above).

When regards to cell volume, primary cells cannot be expanded in culture to provide a reliable biomass that is readily available. As an approximation, it has been suggested that the PHH yield required from hepatocyte elutriation is threefold greater than the number required for experimental purposes due to cells lost during processing (although this figure includes cryopreservation in the processing stages [114]. Pooling allogeneic hepatocytes (frequently done in functional studies) is possible, but is logistically more complicated and would theoretically introduce an increased immunological risk to the patient. Freezing procedures for PHH are inefficient, cell viability is often lost upon thawing, and this also restricts the utility of PHH.

There are also functional limitations to the use of PHH within a BAL. Loss of transcription of liver-specific genes occurs during isolation and subsequent culture: Cytochrome P450 function declines by up to 50% per day in culture and stabilises at around 10% of initial activity [115]. Loss of cell-cell contacts results in greater decreases in CYP expression [116]. CYP expression in PHH can be maintained through culture on ECM (such as collagen) or in 3D format using adapted media [110, 111, 117-120], however, as discussed in section 1.4.2, use of certain culture matrices and media supplements are restricted by BAL compatibility.

With regard to CYP expression, primary human hepatocytes can be considered as a 'gold standard comparator' but are unsuitable for use in the LG BAL due to availability constraints and their inability to sustain function.

1.5.2 Porcine Hepatocytes

Porcine hepatocytes are readily available in large quantities and have been trialled with some success within the Amsterdam Medical Centre Bioartificial Liver (AMC-BAL) [121]. Although bioreactor systems loaded with porcine hepatocytes were demonstrated to be less stable than those loaded with human ones [122], this has been addressed by immortalisation in the HepLui Porcine hepatocyte cell line, albeit (like in human cells) with some compromise in CYP function [123, 124].

Species specific differences are associated with metabolic incompatibility, particularly with regard to xenobiotic clearance. In spite of an inter-species discrepancy in CYP3A induction (CYP3A expression in pigs is induced by rifampicin but not by dexamethasone), porcine hepatocytes are considered to be a relatively good experimental model for human CYP3A function (including testosterone, nifedipine and midazolam metabolism) [35, 36, 38, 125-127]. Also, CYP expression in porcine hepatocytes could be considered less susceptible to variation caused by genetic and environmental influences that manifest in hepatocytes of human origin.

In spite of the (limited) positive results obtained with porcine BAL systems, their use is largely considered unsuitable since xenotransplantation regulations in many European countries restrict the use of porcine hepatocytes in clinically applied BAL systems [105];

xenogeneic cell lines are associated with protein incompatibilities and impart a risk of zoonoses, including porcine endoretroviruses (PERVs) and Hepatitis E [128-130]. Therefore cell lines of human origin are the long term viable option in BAL development.

1.5.3 Human Hepatocyte Cell Lines

Human hepatocyte cell lines could be used to obtain sufficient cell quantities for BAL use, although identifying a cell line that retains both proliferative capacity and hepatic function remains challenging. Hepatoma derived cell lines offer proliferative capacity but do not conserve normal hepatocellular properties due to gene silencing/ dedifferentiation. Additionally, hepatoma derived cell lines present a risk of tumorigenesis in the patient. Immortalisation of human hepatocytes results in better conservation of function, but this is at the cost of proliferation; increased proliferation leads to concomitant loss in function and carries a risk of oncogenic transformation; hence achievable biomass size is limited.

1.5.3.1 Hep G2 Cells: The Cell Line of Choice for the Liver Group BAL

The human hepatocellular carcinoma cell line Hep G2, isolated from a liver biopsy sample from a 15 year old Caucasian male, has been extensively characterised and is the hepatocyte cell line of choice for The Liver Group (LG) BAL. A subclone of HepG2 cells, C3A, has been used in a BAL to treat patients in a pilot-controlled trial [131] and, although no improvement in survival was observed in the hollow fibre based Extracorporeal Liver Asist Device (ELAD), this sets a regulatory precedence for use of HepG2 cells in a device to treat humans. A modified ELAD system using C3A cells (VitaGen ELAD®) is currently undergoing further clinical trials.

HepG2 is a well characterised cell line that retains many hepatocyte characteristics compared to other hepatoma-derived cells [132]. It has been demonstrated that 3D culture of HepG2 cells results in the formation of cohesive spheroids leading to increases in hepatocyte specific functions, such as synthesis of albumin and clotting factors [109, 133, 134]. Moreover HepG2 cells remain viable and retain their metabolic and synthetic capacity during liver failure plasma perfusion [135].

Drawbacks of HepG2 cells include a lack of ammonia metabolism and other limited levels of detoxificatory functions, including Cytochrome P450 activity, observed in monolayer culture.

1.5.3.1.1 Xenobiotic Metabolism in HepG2 Cells

Reports of CYP expression in HepG2 cells are often conflicting. Some authors report no CYP3A4 expression in HepG2 cells; others have demonstrated inducible CYP3A function in these cells [20, 136]. It has been proposed that CYP activity in HepG2 cells is source dependent [51] and that CYP function (or lack of) in HepG2 cells is influenced by a number of factors including culture media and passage number [137-139].

As a general consensus, transcripts of those CYP isoforms involved in drug metabolism are present at HepG2 cells but at levels lower than measured in PHH. As a result, under basal conditions in monolayer culture, Cytochrome P450 function in HepG2 cells is virtually undetectable [20, 45, 136].

Reasonable levels of CYP1A function can be achieved in HepG2 cells by chemical induction [139]; CYP1A activity alone is probably insufficient for a BAL since in humans it accounts for less than 10% of xenobiotic metabolism and in many instances may result in procarcinogen production [42]. Transcript levels of CYP3A4 in HepG2 cells are far lower (100-1000 fold) than in adult PHH [136, 139]. CYP3A7 expression in HepG2 cells has been measured at levels approaching adult primary human hepatocytes [20] but, as described in section 1.2.4.2, the contribution of CYP3A7 to CYP3A expression and function in adult PHH is minimal. This implies that the level of HepG2 CYP3A7 expression is insufficient to provide CYP3A function within a BAL.

Expression of enzymes involved in Phase II metabolism are more in accordance with those found in PHH. Levels of SULT1A1, 1A2, 1E1, mGST-1, GST μ1, NAT1 and EPHX1 in HepG2 cells are similar to or exceed those measured in PHH and GST enzyme activity in HepG2 cells was approaching levels in PHH. As an exception, UGT expression and activity is compromised in HepG2 cells [18].

Overall, there is an imbalance between Phase I and Phase II metabolism in HepG2 cells. This could be addressed by improving CYP expression in HepG2 cells, which could be achieved in a number of ways.

i) Adaption of Culture Conditions to Improve HepG2 CYP Function

In HepG2 cells, modified culture conditions can alter hepatocyte specific functions. In the case of C3A cells, parental HepG2 cells were adapted to different culture medium, which had the apparent effect of increasing urea production[131]. WGA cells were derived from HepG2 cells by metabolic selection involving culture in medium containing fructose in place of glucose. These cells have a higher level of CAR and CYP2B6 which is inducible by phenobarbital [140]. Induction of CYP3A4 function in HepG2 cells has also been achieved by co-culture with bovine endothelial cells [141].

ii) Genetic Manipulation of Hep G2 Cells: Cytochrome P450 Isoforms

The construction of a stable HepG2 derived CYP containing cell line would lend itself to a designer cell line approach for the bioartificial liver device. Transient transfection of single CYP isoforms results in high levels of CYP function in HepG2 cells[142]. Other laboratories have previously produced HepG2 cell lines which stably express single isoforms of CYPs [143-146], however these cell lines had a low metabolic activity relative to PHH; sustained function of multiple CYP isoforms at a level high enough for use in a BAL is difficult to attain.

iii) Genetic Manipulation of HepG2 Cells: Transcription Factors and Nuclear Receptors

Creation of stable CYP transfectants does not address deficiencies in mediators of CYP expression in HepG2 cells: nuclear receptors (NR) and liver-specific transcription factors are expressed at lower levels in HepG2 cells. Increased HepG2 expression of transcription factors, including HNF-4 and C/EBPα or nuclear receptors including PXR, CAR and GR, results in upregulated CYP function [44, 147-152]. NR reporter gene assays are frequently used by the pharmaceutical industry as part of the drug discovery process [153]; potentially this could provide a means to introduce HepG2 CYP3A function within a BAL.

1.5.3.2 Alternative Hepatocyte Cell Lines for BAL Use

Recently, a number of immortalised human hepatocyte cell lines have been developed with reported CYP3A activity. Selected cell lines are compared in Table 1-2.

Table 1-2. Recently developed hepatocyte cell lines with CYP functionality demonstrated by Western blot and/or mRNA analysis with functional assay.

Cell Type	Origin	Culture Format/ Recommended Media	CYP Function/ Nuclear Receptors	References
Fa2N-4	12 year old female Immortalisation following SV-40 transformation of PHH culture	Collagen substratum Multicelltech proprietary medium (MFE™)	CYP1A2, 2C9, 3A4 AhR, PXR	154-156
HepaRG	Adult female Hepatocellular carcinoma derived	2% DMSO Williams' E Medium + 50μM hydrocortisone	CYP1A2, 2C9, 2D6, 2E1, 3A4 AhR, PXR, CAR, PPARα	157-159
HC-04	Spontaneously immortalised from normal liver obtained following hepatoma resection	Fibronectin and collagen matrix Cambrex Hepatocyte Culture Medium (HCM) Bulletkit	CYP1A2, 2C9 2E1, 3A4 PXR, AhR	160, 161

Although these cell lines demonstrate CYP functionality, they may not necessarily be suited to large scale BAL culture. In addition to being able to perform drug metabolism functions in standard monolayer conditions, these cells must be able to function in BAL specific medium, and survive bioreactor culture.

HepaRG cells only differentiate into hepatocyte-like morphology following treatment with 2% DMSO [162] and HC-04 and Fa2N-4 cell lines are both cultured on collagen matrices which are unsuitable for use in extracorporeal circuits. Furthermore CYP function of these cell lines may be insufficient (based upon reported literature values). CYP3A4 protein expression can be increased in HC-04 cells by the PXR agonist rifampicin and these cells are able to metabolise the CYP3A substrate midazolam, however their drug metabolising capacity is equivalent only to PHH from donors with low levels of CYP3A4 function [160].

Fa2N-4 cells demonstrate both CYP1A2 (EROD) and CYP3A4 (testosterone, midazolam) activity, but their levels of function are less than 75% of PHH. Expression levels of mediators of CYP1A2 (AhR) and CYP2C/3A (PXR) induction in Fa2N-4 cells match those found in PHH but their reduced drug metabolism capacity could partially be explained by a deficiency in the CYP2B/CYP3A mediator CAR and by decreased expression of hepatic uptake transporters [154, 155].

1.5.4 Human Foetal Hepatocytes for BAL Use

Human foetal hepatocytes (FHH) are expandable and express relevant drug metabolising enzymes that are upregulated by 3D culture [163], so may be a considered as a source of CYP3A function within a BAL. FHH differ from adult PHH in their preferential expression of CYP3A7 to CYP3A4 and in this respect are often compared to HepG2 cells which are widely reported to share this characteristic [20, 138, 164]. As in adult liver, there is a high level of interindividual variability in both CYP and NR expression in FHH [165].

1.5.4.1 Ontogeny of Human Hepatic CYP3A

In FHH, variation in CYP expression can in part be attributed to ontogeny. Whilst some report a ‘developmental switch’ at birth between the two isoforms [166], others more

recently have reported a 'developmental transition' [53, 167]. The latter author described a study that examined CYP expression in human liver samples obtained from 8 weeks gestation to 18 years of age. CYP3A7 expression was found to be high in foetal liver and decreased after birth (although continued to be the predominant CYP3A isoform in the neonate until several years after birth), whereas CYP3A4 expression was low in the foetal liver and remained so at birth and during the neonatal period; increases in human liver CYP3A4 expression are not achieved until several years after birth.

1.5.4.2 Role of CYP3A7 in FHH

The dominant expression of CYP3A7 in the foetus could be attributed to its role in foetal development [168]. CYP3A7 is central to the role of placental estriol biosynthesis (important to foetal growth and parturition) and CYP3A7 specific catalysis also has a protective role, preventing foetal exposure to steroids and retinoic acid which may influence proliferation and differentiation. CYP3A7 has also been implicated in promutagen activation [169], although the determinants of CYP3A7 expression in HepG2 cells are less clearly defined.

1.5.4.3 FHH Mediated CYP Metabolism within a BAL

As described above, whilst expression of CYP3A4 in FHH is lower than in adult cells, CYP3A7 expression is increased. Potentially this could provide a means to CYP3A mediated metabolism within a BAL. In order to address the issue of limited supply, immortalised foetal liver cell lines could also be considered [170, 171].

When evaluated within the AMC-Bioreactor, CYP function (lidocaine metabolism) of the immortalised foetal hepatocyte cell line cBAL111 was lower (>99%) than levels measured in adult PHH [171]. In contrast, FHH loaded into a laboratory scale (10x downscale; 55ml) bioreactor consisting of a perfused 3D culture system were able to metabolise lidocaine, indicating CYP3A (and CYP1A2) function; moreover extent of elimination was 3.5 fold greater than in mature porcine hepatocytes and 6.6 fold greater than adult primary human hepatocytes [122], though this may be due to the phenotypic instability of the primary adult cells. The use of FHH within a BAL will be discussed further in Chapter 3.

1.5.5 Stem Cells for BAL Use

Stem cells regenerate themselves through mitotic cell division and generate specialised cells through differentiation and so have the potential to offer an unlimited hepatocyte cell supply for a BAL. A number of stem cell-like cells that can differentiate into hepatocyte-like cells have been identified which could act as potential surrogates to provide liver functions within a BAL. The benefits and limitations, including their CYP activity, of four cell lines of differing stem cell origin will be discussed below.

1.5.5.1 Human Embryonic Stem Cells (hESC)

Cytochrome P450 function was measured in hepatocyte-like cell lines (SA cells) differentiated from hESC by 18-30 day treatment with basic fibroblast growth factor (bFGF) [172]. Gene expression (mRNA) was compared between PHH, HepG2 and SA cell lines. Both CYP1A2 and CYP3A4/7 expression were lower in SA cells than PHH; the discrepancy in CYP3A expression between PHH and SA cells was greater for CYP3A4 than CYP3A7 and there was greater expression of CYP3A7 than CYP3A4 in the latter cell type. CYP1A2 expression was higher in SA cells than in HepG2 cells, CYP3A4/7 expression was generally comparable between HepG2 cells and SA cell lines. Both CAR and PXR mRNA expression were present in SA cells.

Western blot analysis was used to quantify CYP1A2, 3A4 and 3A7 protein expression in SA cells and PHH. Presence of all 3 proteins was detected in SA cells and their expression was increased by chemical induction, but expression levels were low compared to PHH and CYP3A/1A function could not be detected in SA cell lines.

In the example described above, hepatocyte-like cells derived from hESC demonstrated expression of relevant CYPs at mRNA and protein level but were unable to provide CYP function. They did however express high levels of a number of hepatic transcription factors including HNF4α, C/EPBα and β, and the increased presence of CYP3A7 relative to CYP3A4 could be indicative of an immature phenotype. Potentially hepatic function could be improved by further differentiation.

The cells characterised in the above study illustrated a practical consideration of stem cell development for BAL use. Proliferation of hESC is generally superior when performed on a feeder cell layer (probably due to cell-cell interaction between the two cell types). SA cells were grown on a layer of mouse embryonic fibroblasts (MEFs) and

contained a low amount (around 12%) of MEF contamination. Contamination of progenitor cells with a feeder cell line, which has also been described by others [173], could have an impact on cell function and (particularly in the case of cross-species contamination) would render cells unsuitable for BAL use. Use of feeder cell lines can be avoided by the use of ECM such as Matrigel but these are of animal origin and also carry a risk of cell line contamination [100, 173].

1.5.5.2 NeoHep Cells

NeoHep cells are generated by transdifferentiation of human peripheral blood monocytes (PBMC) obtained from healthy human donors. They were developed by culturing PBMC in dedifferentiation medium to generate "programmable cells of monocytic origin" which were then induced to hepatic differentiation by 14 day treatment with fibroblast growth factor-4 (FGF-4) [174, 175]. They resemble PHH with respect to their phenotype (although are 2-4 times larger), express hepatocyte markers and a range of hepatocyte specific functions including albumin secretion, urea production, lactate production and phase I and phase II metabolism.

CYP1A2 and CYP3A4 mRNA expression was quantified in NeoHep and both isoforms were shown to increase during differentiation, however CYP1A2 and CYP3A4 mRNA expression was lower (62 and 83%) than in PHH. CYP3A4 mRNA expression in NeoHep cells was also decreased relative to HepG2 cells. Both CYP1A2 and CYP3A4 proteins were present in NeoHep and were inducible with 3-MC or rifampicin respectively; however level of expression was lower than measured in PHH.

Function of CYP1A and CYP3A4 was also quantified in NeoHep cells under basal conditions and, in accordance with their mRNA and protein expression, was decreased relative to PHH [175, 176]: CYP1A1/1A2 function was ~9 fold lower in NeoHep cells although the fold induction values measured between PHH and NeoHep cells were comparable, hence function remained lower in the latter cell type. CYP3A4/CYP1A2 function was ~ 5-fold lower in NeoHep compared to PHH but the fold induction between PHH and NeoHeps were comparable [177, 178].

Testosterone metabolism was also measured in NeoHep cells to determine CYP3A4 function. Following rifampicin induction their production of 6β-hydroxytestosterone

was ~ 1000 times less than induced human PHH and ~10-times less than vehicle treated PHH maintained in culture [175].

Interestingly, CYP expression varied in NeoHep cell lines which were developed from PBMC from different donors. This demonstrates that interindividual variation in CYP expression described in section 1.2.5 is conserved at the stem cell level. Practically this phenomenon could be addressed by using PBMCs pooled from multiple donors and selection of high-activity cell lines for expansion. Function of NeoHep cells is insufficient for BAL; potentially this could (like hepatocyte cell lines) be increased by bioreactor culture. Considerations relevant to this would include the stability of these cells during mass expansion and prolonged culture. Use of growth factors during this process would be a prohibitive factor for large-scale bioreactor culture.

1.5.5.3 Human Hepatoblasts from Foetal Hepatocytes

Hepatoblasts are hepatic stem cells in foetal liver which develop to mature hepatocytes during foetal liver development and have been considered for BAL use [179]. Sodium butyrate (SB) was used to induce hepatoblast formation from foetal hepatocytes. These cells were then cultured on hydroxyapatite microcarriers (derived from a biocompatible mineral species which lends itself to artificial organ use) in order to produce high density cultures for bioreactor use. Under high density culture conditions, following SB differentiation, hepatoblast cells had improved CYP function which was induced by rifampicin and measured by benzyloxyresorufin-*O*-dealkylation activity. However in preliminary experiments, proliferation on a clinically applicable scale was not achieved, and CYP3A4 function was not characterised any further.

1.5.5.4 Human Adult Liver Resident Stem Cells (HLSC)

Human liver stem cells have been identified in adult liver that have self regenerating capacity and are multipotent [180]. In adhesion, these cells differentiate into mature hepatocytes only after the addition of growth factors (HGF, FGF4). By using a hollow fibre rotary BAL, differentiation can occur without the addition of growth factors and, when cultured in this manner, Cytochrome P450 activity is increased (approximately three-fold) relative to adhesion cultures [181] which supports a hypothesis that Cytcohrome P450 function is favoured by a 3D culture environment. Achieving stem cell differentiation in this manner could abrogate the use of exogenous growth factors which would provide a more economic means of achieving BAL scale biomasses of stem cells.

1.5.5.5 Stem Cell Conclusions

With respect to the use of stem cells within a BAL, challenges include achieving sufficient function relative to adult PHH, achieving unlimited proliferation and controlling differentiation to achieve relevant levels of hepatic function without the use of culture matrices or exogenous growth factors. Expression of various isoforms of CYP enzymes occur at different stages of differentiation [182]: CYP 1B1 and 2E1 proteins and mRNA have been detected in cells at early stages of differentiation, but CYPs 3A4, 3A7 were 2C9 were absent. Additionally, phenotypic and genotypic stability *in vitro* must be maintained in bioreactor culture in order to generate large biomasses [98]. Beneficial effects of bioreactor culture on CYP expression and function have been shown for human stem cells however these need to be demonstrated for longer term cultures on a clinically applicable scale.

1.5.6 Alternative Cell Lines for a Co-Culture Approach

Within a BAL, it may be feasible to enhance or supplement hepatocyte function through the use of a dual cell approach and there are two possible options for this.

i) Co-culture to enhance existing CYP function

Heterotypic cell interactions are absent from pure hepatocyte cultures and, when included as part of a 3D culture environment, may aid in the maintenance of CYP function by improving hepatocyte viability and maintaining differentiation. This has been demonstrated in primary hepatocytes of both rat and human origin using both allogeneic (human cells from multiple donors) and xenogeneic (rat and mouse) cells of hepatic and non-hepatic origin. Examples of cell types which result in improved hepatocyte CYP function in co-cultures include, but are not limited to, stellate cells, endothelial cells, biliary epithelial cells and fibroblasts [110, 141, 183-185].

Co-cultures of the above nature improve ECM deposition and cell-cell contact, which in turn improves hepatocyte specific functions in PHH such as CYP activity. However, influence of these co-cultures on HepG2 function may be less beneficial since HepG2 cells themselves are able to produce many ECM proteins and form cell-cell contacts in 3D culture. In addition, issues associated with cell line contamination would need to be addressed.

ii) Non-liver cell lines to provide a detoxificatory component

As described in section 1.2, Cytochrome P450 drug metabolising functions are not restricted to the liver and the expression and function of these enzymes has been considered in many non-hepatic cell lines. Potentially a non-liver cell line could be cultured alongside HepG2 cells to provide CYP3A function within the LG BAL. (Arguably) cell lines derived from the gastrointestinal (GI) tract are the most promising option since CYP3A4 and associated transcription factors are expressed in the GI mucosa and contribute heavily to first-pass metabolism at this site [186, 187].

A study was carried out by Schmiedlin-Ren *et al.* [188] to compare CYP expression in monolayer cultures of primary human hepatocytes with cell lines derived from tissues known in man to express CYP3A enzymes. 1α, 25-dihydroxyvitamin D3 (VD3) mediated CYP3A4 induction was observed in Caco-2 (colon), LS180 (colon), and HPAC (pancreas) cell lines. In the monolayer culture conditions used by the authors (laminin ECM with optimised differentiation medium), CYP3A4 enzyme expression and substrate metabolism were considerably lower in the cell lines than in the primary cultures. This may be a significant limitation to the use of these cells within a BAL, although the effects of 3D culture on CYP3A function in these cell lines has not been widely explored. This will be addressed further in Chapter 5.

1.6 The Liver Group BAL

The biomass of the current LG BAL comprises HepG2 cells encapsulated in alginate (Figure 1-6). Alginate forms a hydrogel which is permeable, provides anchor support and allows cell proliferation in 3D format. Patient plasma is perfused through the beads and this allows direct contact with the spheroids, and therefore exchange of large proteins between plasma and spheroids, which is not restricted by mass transfer membranes used in some devices.

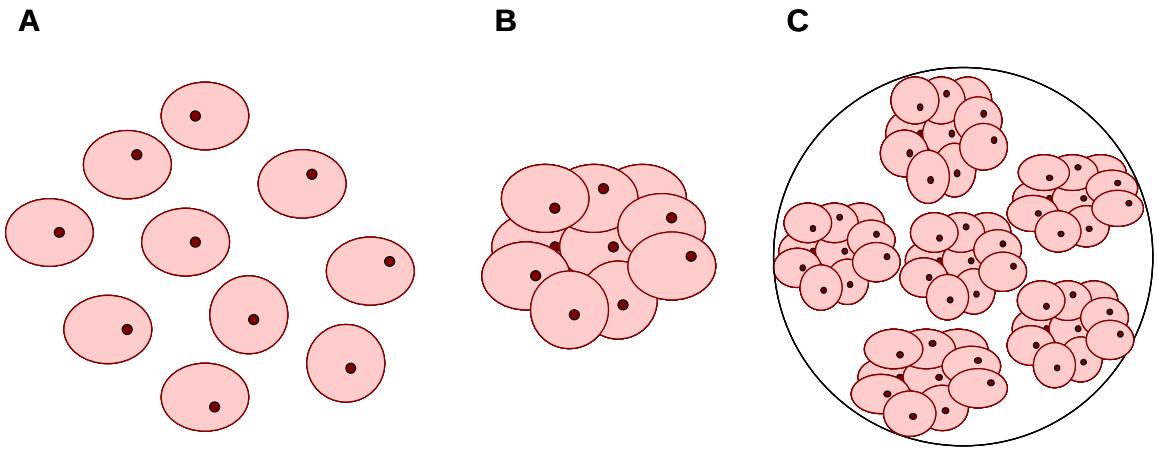


Figure 1-6. Following alginate encapsulation of a single cell suspension (A), multicellular colonies or spheroids (B) form within alginate beads (C). Typically, following 8 days of static culture, a 450µM bead contains 20-25 spheroids and each spheroid contains 8-20 HepG2 cells [189].

The BAL process would begin by expanding HepG2 cells in monolayer culture prior to encapsulation in alginate at a seeding density of $1.5x10^6$ cell/ml beads. The beads would be cultured in a fluidised bed bioreactor (FBB) using a medium optimised for maximum growth and differentiation. FBB culture provides a microgravity environment allowing high mass transfer between beads and medium without shear stress and improved cell proliferation within the beads thereby increasing bead performance. Parameters including flow rate, oxygenation, temperature and pH can also be also tightly regulated within the bioreactor circuit to provide optimal conditions. Cells would be cultured for 8-10 days to generate at least a third of the total cell numbers found in a 70kg male human liver (7×10^{10} hepatocytes).

The LG BAL would be a dual circuit device. In the first circuit, plasma is isolated from the patient by plasmapheresis and then passed to the secondary circuit, comprising

HepG2 cells held within the FBB, where it is purified by recirculation through the biomass. Plasma is then returned to the primary circuit via a quality management system which is included for the removal of cell debris and DNA.

So that the device can be used as a bedside treatment in an emergency situation, the cartridge holding the cultured beads would ideally be cryopreserved for long-term storage.

1.6.1 Considerations Relevant to the Use of Cell Lines in a Bioartifical Liver Device

As described in previous sections, there are several existing cell sources available that exhibit CYP3A function, however in order to be fit for purpose, these cells must be able to fulfil regulatory requirements and be easily adaptable to BAL relevant culture conditions whilst maintaining a high level of function.

1.6.1.1 Regulations Relevant to the Development of a BAL for Clinical Use

A device intended for use in treating humans must comply with healthcare regulations stipulated by governing bodies such as the MHRA (Medicines and Healthcare products Regulatory Agency, UK), the FDA (Food and Drugs Administration, USA), the EMEA (European Medicines Agency) and the JPAL (Japanese Compliance and Medical Approval Agency). The requirements of these agencies are by no means unified [190], however to attain multi-market approval, the following components should be considered.

Cell Source: immortalised cell lines should not contain active retroviral elements. Tumor derived cell lines should not carry risk of tumorigenesis. Any cell line should have traceable origins and also be homogenous and free from contamination (potentially caused by components such as feeder cells, murine viral elements from Matrigel matrices or adventitious microbial elements).

Culture Matrix: alginate is the selected culture matrix for the LG BAL. It was originally purified from seaweed, is bio-compatible, easy to manipulate and can be cryopreserved. Importantly, is already widely used within the food industry and in the development of pharmaceuticals and wound dressings. Alginate purity, although outside the scope of

this thesis, has been considered by others during LG BAL development. To be used within the LG BAL any cell line should be compatible with this culture matrix: proliferation, function and viability of selected cell types should not be adversely affected by alginate encapsulation.

DNA/Cell Debris Transfer: biological contaminants from the biomass should be controlled, purified and eliminated. As described, DNA and cell debris transfer will be controlled and monitored by a Quality Management System. Cell viability will have considerable impact on biomass integrity and so should be maintained at a high level within bioreactor culture.

1.6.1.2 Cryopreservation and Storage

To allow a BAL to be used routinely in clinical applications, a long-term storage method must be developed. This will most likely involve cryopreservation.

The effect of cryopreservation on Cytochrome P450 function has been studied in both animal and human hepatocytes [191-194] and has been shown to be minimal; hepatocytes appear to maintain their CYP function subsequent to cryopreservation (albeit with a slight decrease in basal activity), and various techniques employed during freezing (such as use of cryoprotectants and progressive freezing) may aid in this endeavour [191, 195].

It should, however, be noted that impact of cryopreservation on CYP function has mainly been considered following freezing of low volume simple cell suspensions. Cryopreservation of encapsulated liver spheroids, particularly on a large scale, bears greater resemblance to the cryopreservation of tissue. The impact of this process (including the influence of components of the solutions used) on Cytochrome P450 function will need to be accounted for.

Short-term storage should also be addressed. When measured *in vivo,* a period of prolonged (5-10 days) 4°C exposure resulted in increased rat hepatic CYP content particularly for CYP1A1/2 [196, 197]. Studies using the AMC-BAL [198] found that detoxificatory function was maintained in porcine hepatocytes following a period of mild hypothermic preservation (15°C for up to 24 hours).

1.6.1.3 Culture Conditions and Media Supplements

Choice of culture media is important when considering CYP function within BAL relevant cell lines and there are both practical and functional constraints. Large volumes of media are required for BAL culture, and so it should not therefore be prohibitively costly. Addition of growth factors can promote hepatocyte differentiation, but these are also expensive for mass culture use and may decrease CYP function; presence of human hepatocyte growth factor had an inhibitory effect on both CYP function and expression for multiple isoforms of CYP1, CYP2 and CYP3 [199, 200].

In primary cultures, different culture media have a demonstrable effect on CYP function (although this effect is species specific) [117, 201, 202]. If culture medium does not support hepatocyte differentiation, there will be a rapid drop in drug-metabolising capacity and there is also compelling evidence that CYP function in HepG2 cells is influenced by culture media [51, 137].

Media supplements, used in hepatocyte studies to improve differentiation, include glucocorticoid agonists (such as dexamethasone, prednisolone, or hydrocortisone) and insulin. These also regulate expression of Cytochrome P450 and nuclear receptors including PXR, CAR and RXRα [55, 95, 96, 203-205].

1.6.1.4 Using Human Fresh Frozen Plasma to Supplement Culture Medium

In order to eliminate the use of animal derived reagents within the LG BAL, human fresh frozen plasma (FFP) has been used in culture medium in place of place of foetal calf serum (FCS). Plasma is pooled from multiple donors in order to minimise donor dependent effects and the use of FFP as a media supplement has been shown to significantly improve LG BAL proliferation (data not yet published). Improved hepatocyte differentiation following culture in human FFP should improve CYP function, however, plasma components including cytokines and free fatty acids could also regulate Cytochrome P450 expression. The possible effects of FFP exposure on CYP induction will be explored in Chapters 3 and 4.

1.6.1.5 The Effect of Liver Failure Plasma Exposure on CYP Function

Since it will be in direct contact with patient plasma, prolonged perfusion of the BAL biomass in liver failure plasma (LFP) could affect its Cytochrome P450 function.

Following perfusion (3-15 hours) of human hepatocyte cell lines in normal human plasma, improved function (relative to culture medium) of some but not all Cytochrome P450 isoforms has been demonstrated [133, 171], however plasma from patients with acute liver failure is potentially cytotoxic and could have further implications for BAL function.

In addition to its pro-apoptopic properties [206], plasma from liver failure patients may potentially contain endogenous and xenobiotic components which could inhibit CYP enzyme function; within a BAL, LFP will be from a single patient and the effects of interpatient variation, arising from the range of clinical and biochemical manifestations that present during acute liver failure, cannot be discounted. This has been demonstrated using monolayer cultures of the immortalised human hepatocyte cell line HHY41 exposed to normal plasma, LFP or culture medium for 16 hours [133]. Dibenzanthracene (DBA) mediated induction of CYP1A was significantly reduced in three out of four LFP samples (determined by a decrease in MROD and EROD activity when compared to control plasma). Two out of four LFP samples significantly decreased the biotransformation of caffeine to its primary metabolites. Steroid (androst-4-ene-3, 17 – dione) metabolism was altered in two out of four LFP samples. EROD and MROD activities were improved following incubation with normal plasma (4 independent samples) relative to culture medium whereas caffeine biotransformation and steroid metabolism were unaffected.

More recently the effect of LFP on CYP function has been examined in an alginate encapsulated HepG2 system. Coward *et al* [135] demonstrated that DBA induced CYP function was maintained in HepG2 cells following a 3 hour exposure to LFP which diminished by around 30% after 6 hours of exposure. In contrast, CYP function was maintained following 6 hour exposure to plasma from healthy donor plasma.

1.7 Experimental Approaches Selected for this Project

As described, existing CYP expression may be upregulated by chemical induction. This may be exploited within BAL relevant cell lines to achieve improved CYP function. Cells will be treated with compounds that are known CYP inducers and their function then measured using a range of bioassays.

1.7.1 Chemical Induction of CYP Function

Inducers that have been shown to increase CYP1A and CYP3A expression were selected and these are shown in Table 1-3.

Table 1-3. Chemical inducers selected for use in this thesis.

Inducer	Chemical Class	CYP	NR	Dimerisation Partner	References
Dibenzanthracene	Aryl hydrocarbon	CYP1A	AhR	ARNT	61
Indirubin	Kinase Inhibitor Endogenous Ligand	CYP1A	AhR	ARNT	207, 208
Dexamethasone	Glucocorticoid	CYP3A	GR/ PXR/CAR	RXRα	95, 96, 204
Rifampicin	Rifamycin antibiotic	CYP3A CYP2C CYP2B	PXR	RXRα	209
Phenobarbital	Barbituate	CYP2B CYP2C CYP3A	CAR	RXRα	209
1α,25-Dihydroxyvitamin D_3	Hormone (Biologically active form of Vitamin D3)	CYP3A	VDR	RXRα	188

1.7.1.1 BAL Relevant Considerations With Respect to Inducers

Both vehicle and inducer must be suitable for use in a device intended for human use. In this thesis, dimethyl sulfoxide (DMSO) 0.1% v/v was selected as a vehicle due to its ability to solubilise all of the selected inducers. Although the concentration of DMSO selected was one that has been previously demonstrated not to have an effect on CYP induction [210-212], DMSO will not be used as a solvent vehicle within the LG BAL. If necessary, an appropriate vehicle would have to be sought depending upon selected inducer, which with the exception of dibenzanthracene (DBA) are therapeutic agents and/or endogenous ligands and so would meet regulatory guidelines in the context of human exposure. DBA is carcinogenic and human exposure should be avoided, therefore would probably be inappropriate for BAL use, but was included in this study as a classical CYP1A inducer with a different chemical class to indirubin which is an endogenous AhR ligand found in human urine, and has been identified as the active component of several Chinese herbal medicines utilised in the treatment of cancer.

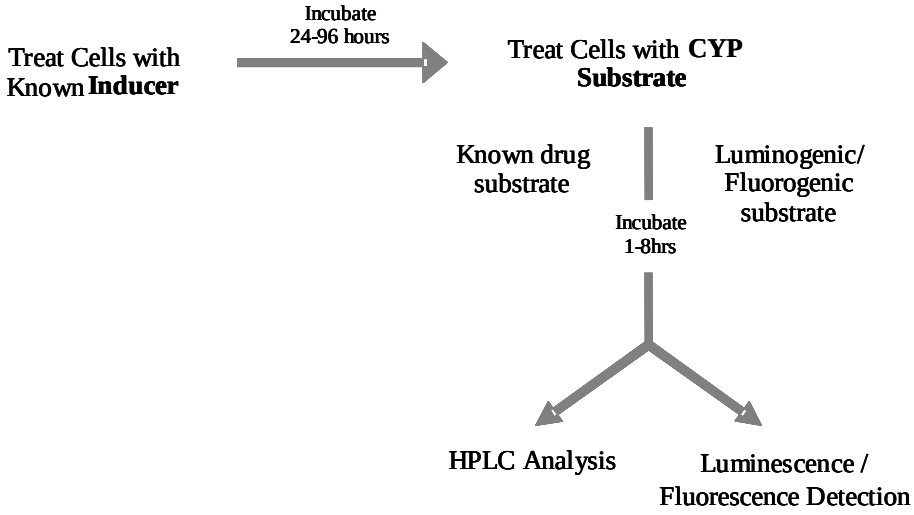
1.7.1.2 Duration of Induction

Chemical induction is a slow regulatory process which may take between 24 and 96 hours to come about. Although this is partly due to the system in which induction is measured (*in vivo*, liver slices, cell culture systems), this time frame is also CYP dependent; it is widely thought that CYP1A induction occurs between 24 and 48 hours post exposure whereas CYP3A induction peaks 48-96 hours post exposure [61, 213]. These time differences are likely due to the different receptors involved in isoform specific induction as described in Table 1-3.

Since inducer will not be present within a BAL while it detoxifies patient plasma, an equally pertinent point to consider is how long the upregulated effect would last in the absence of inducer. Although there is little *in vitro* data available, a consensus based on *in vivo* data derived from humans is that it takes time for enzyme function to return to basal levels following induction. Upregulated CYP1A function was measured up to 5 days following aryl hydrocarbon exposure and CYP3A mediated verapamil metabolism was increased above baseline for up to 2 weeks in patients following 1 week of rifampicin treatment [37, 63].

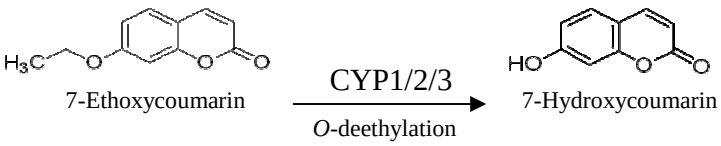
1.7.2 Methods of Quantification

Chemical induction of Cytochrome P450 function requires existing expression in relevant cell lines. The aim of induction experiments will be to increase and subsequently quantify CYP function, as demonstrated by the following schematic:



1.7.2.1 Broad Spectrum CYP Activity

7-Ethoxycoumarin (7-EC) is metabolised by Cytochrome P450 to produce 7-Hydroycoumarin (7-HC), which can then be extracted and quantified by fluorescence; any increases in CYP activity directly correlate to an increase in fluorescent product formation.



7-EC is often described as a "general" CYP substrate since it has been demonstrated that multiple enzymes in the CYP1, CYP2 and CYP3 families are active catalysts of 7-ethoxycoumarin *O*-deethylation (ECOD). It can thus be used as a method of detection when an index of broad spectrum CYP activity is required [214].

1.7.2.2 Identifying Activity Within a Specific CYP Family

1.7.2.2.1 Testosterone Metabolism

Metabolism of testosterone (TST) by Cytochrome P450 can be measured by HPLC coupled with UV detection. Different CYP isoforms are involved in regioselective TST oxidations and in humans, the relative contribution of each CYP to testosterone metabolism has been measured using individual isoforms expressed in baculovirus infected insect cells [52]. CYP3A isoforms are the most important in forming the major metabolites of testosterone and are responsible for 94% of all metabolite production. Further to this, 6β-hydroxytestosterone (OHTST) accounts for 86% of all metabolite production and the majority (84%) is produced by CYP3A4 > CYP3A5 > CYP3A7. However with respect to other metabolites, 2α-OHTST is produced as a minor metabolite of testosterone by CYP3A7 to a greater extent than by CYP3A4 (16 fold). It is also important to note that although testosterone may be metabolised by CYP1A (CYP1A1>CYP1A2) to produce the 6β-OHTST metabolite, it has 60 fold and 1.3 fold lower activities than the CYP3A4 and CYP3A7 isoforms respectively. Measurement of testosterone depletion and production of 6β-OHTST will therefore primarily provide a measurement of CYP3A4 function, however depletion of testosterone may also be indicative of other CYP function.

1.7.2.2.2 Promega P450-Glo™ Assays

Luminesence based assays are a relatively fast and sensitive method of measuring CYP activity. Promega P450 Glo™ assays use luminogenic substrates which are inactive until metabolised by CYP enzymes to luciferin, which then reacts with an additional luciferase reagent to produce an amount of light proportional to CYP enzyme activity. All substrates have the same core structure but each has a derivitisation which confers its selectivity for individual CYP isoforms.

Luciferin ME and Luciferin BE were originally chosen to measure CYP1A2 and CYP3A activities in this thesis. Luciferin PFBE was used in some later studies to confirm CYP3A function. The structures and selectivity of these substrates are shown in Figure 1-7 and are summarised below:

Luciferin ME was developed as a CYP1A2 substrate but was later found to cross react with other CYP isoforms, most notably CYP2C8/9 (involved in xenobiotic metabolism) and CYP4A11 which is involved in arachidonic acid conversion in control of hypertension and fatty acid metabolism. Others have demonstrated that CYP2C metabolism by this substrate can be inhibited by 1µM sulfaphenazole [136].

Luciferin BE was the original luminogenic CYP3A substrate developed by Promega. It is metabolised by CYP3A4/5/7 and also cross reacts with CYP4F12 which is expressed in human liver. The exact function of this isoform is undefined but it is active towards arachidonic acid and prostaglandin H2 and is implicated in antihistamine metabolism.

Luciferin PFBE was developed for use in cell based assays. It is also metabolised by CYP3A4/5/7 but has lower background signal and does not react with CYP4F12.

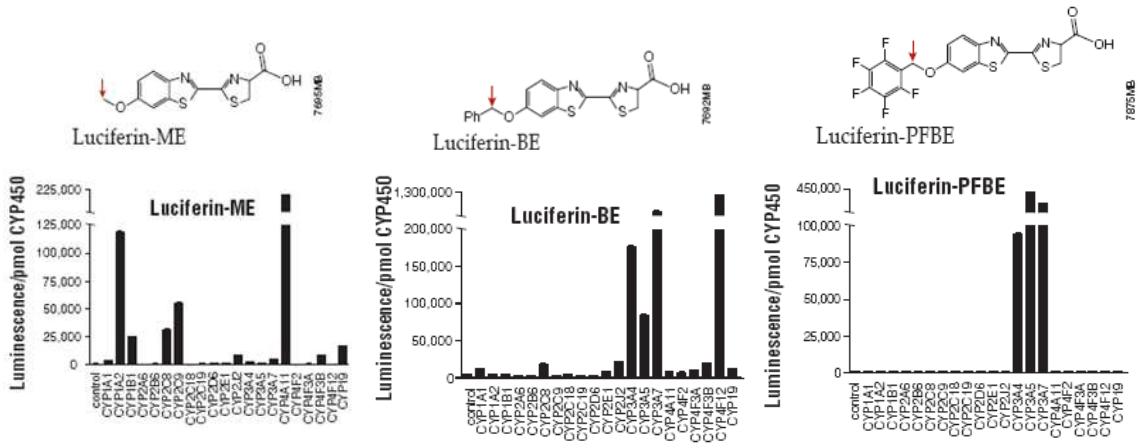


Figure 1-7. Luminogenic P450-Glo substrates are converted to luciferin by CYP isoform spcific mediated catalysis. (top) Isoform specificity of substrates is determined by a derivitization upon the core structure (→) that arises from beetle luciferin. (bottom) Substrate specificity for individual CYP isoforms was measured using recombinant CYP enzymes heterologously expressed in insect cell microsomes. Images were all obtained from [216].

1.7.2.2.3 Invitrogen Vivid ® CYP450 Substrates

Invitrogen Vivid ® CYP450 screening kits were developed to assess metabolism of human CYP enzymes involved in drug metabolism. The substrates are metabolised by specific CYP isoforms to a fluorescent metabolite which can be excited in the visible light spectrum. Vivid ® BOMFC (benzyloxymethylfluorescein) is metabolised by CYP3A and CYP2B6. Substrate metabolism can therefore be measured in the presence or absence of inhibitors to indicate CYP3A or CYP2B6 function; in this thesis clarithromycin will be used to inhibit CYP3A but not CYP2B6 function [54, 217].

1.8 Aims and Hypothesis

The aim of the following work was to identify and characterise a suitable cell source to provide Cytochrome P450 function within a BAL.

As described, CYP3A is the most abundantly expressed subfamily in adult human liver and responsible for the metabolism of the largest range of xenobiotics and is therefore deemed essential for a BAL. Hep G2 cells, used within the LG BAL, are generally not believed to express this subfamily at a functional level; however CYP1A function has been proven in these cells.

Hypothesis: When measured *in vitro*, Cytochrome P450 activity in human cell lines is dependent on culture conditions. Existing CYP expression can be improved by chemical induction and 3D culture which will result in improved function. It can also be increased by genetic manipulation.

Overall, the aims of this thesis were to:

1.) **Determine an appropriate gold standard comparator from preparations of human liver**

CYP function will be measured in adult PHH isolated from a number of donors in order to assess the range of function in human liver. In order to obtain a broad representation of CYP activity in an adult human liver which differentiates between effect of culture conditions and interindividual variation, CYP activity will also be examined in human liver microsomes (HLM) isolated where possible from tissue and hepatocytes from the same donor. This will provide independent confirmation of variation in CYP activity discounting effects of hepatocyte isolation and culture.

In foetal cells the predominant CYP3A isoform is CYP3A7 and in this respect they are often compared to HepG2 cells. Basal and inducible CYP activity will therefore also be considered in these cells.

2.) **Characterise existing CYP activity in Hep G2 cells considering the effect of adaptation to BAL culture conditions**

When cultured in simple culture media HepG2 cells do not achieve CYP mediated drug metabolising functions at an appropriate level; however CYP function in HepG2 cells may depend both on source and culture conditions. Through chemical induction, 3D culture and adaptation of medium components it will be determined how our current BAL culture conditions, which were developed to improve hepatocyte differentiation, impact on CYP activity in HepG2 cells.

3.) **Investigate suitable cell lines that could be adapted to act as the detoxificatory component of a BAL to provide CYP3A function**

Intestinal cell lines which express CYP3A could be treated with inducers to provide CYP function within a BAL. Two different cell lines, Caco2 and LS147T, were selected. Alternatively immortalised hepatocyte cell lines from normal liver may exhibit superior CYP function to HepG2 cells. The human hepatocyte cell line HC-04 was selected due to its expression of CYP proteins and its ability to metabolise the CYP3A substrate midazolam.

The adaptability of these three cell lines to large scale 3D culture will be investigated to determine their ability to both proliferate and to provide CYP functions under these conditions.

4.) **Create, by genetic manipulation, an inducible HepG2 cell line suitable for use in a device to treat humans**

It has previously been reported that decreased mRNA expression of CYPs is responsible for low functional activities of these enzymes in hepatoma cells; therefore improving HepG2 cell expression of CYP mRNA could improve enzyme function.

CYP induction is regulated by nuclear receptors. The expression of nuclear receptors will be improved in HepG2 cells by genetic manipulation and this, in conjunction with chemical induction could improve CYP function in HepG2 cells to provide function comparable to that measured in primary human hepatocytes.

CHAPTER 2 General Methods

Cell culture reagents were obtained from Invitrogen. All other reagents were obtained from Sigma Aldrich unless otherwise stated.

2.1 Mammalian Cell Culture

2.1.1 Preparation of Collagen Type I from Rat Tail Tendons

Collagen type I was prepared from rat tails and applied to tissue culture plates to promote hepatocyte monolayer culture.

Materials

Rat Tails (5 will yield ~ 2g of collagen)
0.01M Acetic Acid
50ml polypropylene centrifuge tubes
Sterile universal tubes
Magnetic Stirrer
Centrifuge

Method

Rat tails were ethanol sterilised and transferred to a class II microbiological safety hood. The vertebrae of the tail from tip to base were sequentially broken using artery clamps, and the central tendon removed after each break. The tendons were weighed and then 300mls of acetic acid per gram of tendon added. The mixture was stirred for 2-3 days at 4°C until the tendon was dissolved and then transferred to 50ml polypropylene centrifuge tubes and centrifuged for 2 hours at 800g. Supernatant was dispensed into sterile universal tubes and stored at 4°C until required.

2.1.2 Collagen Coating of Cell Culture Plates

Materials

12-well tissue culture plates

Sterile HBSS without calcium and magnesium

Sterile saline

Collagen type I (prepared from rat tail)

UV Lamp

Method

To a 12 well plate, 1 ml of collagen per well was added and left for 5 minutes at room temperature. Following this time, excess collagen solution was vigorously flicked from the wells which were then rinsed twice with 1ml of HBSS. 1ml of sterile saline solution was added to each well and the plate irradiated without the lid under short wave UV light for 15 minutes. Each plate was used on the same day as preparation.

2.1.3 Preparation of Culture Media

During media preparation, all supplements were pooled into a 50ml centrifuge tube and filter sterilised prior to addition to culture media.

2.1.3.1 Primary Adult Human Hepatocyte Media (AHM)

Phenol Red-Free Williams' Medium E (Sigma W4128)
2mM L-Glutamine
100U/ml Penicillin/0.1mg/ml Streptomycin
1.25μg/ml Fungizone
10mM Nicotinamide
10nM Dexamethasone
5.8ng/ml Insulin (Actrapid Novonordisk)
1mM Ascorbic Acid-2 Phosphate
50μM (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox)

2.1.3.2 Human Foetal Hepatocyte Medium (HFM)

Phenol Red-Free Williams' Medium E
20mM HEPES
2mM L-Glutamine
100U/ml Penicillin/0.1mg/ml Streptomycin
1.25µg/ml Fungizone
10mM Nicotinamide
200µM Ascorbic Acid-2 Phosphate
100nM Dexamethasone
6.25µg/ml Insulin
6.25µg/ml Transferrin
6.25ng/ml Sodium Selenite
5.35µg/ml Linoleic Acid BSA
550µg/ml Pyruvate

2.1.3.3 Hep G2 Culture Medium (Complete Culture Medium)

α-MEM with ribonucleosides and deoxyribonucleosides (Invitrogen 32571)
10% Foetal Calf Serum (FCS)
2mM L-Glutamine
100U/ml Penicillin/0.1mg/ml Streptomycin
1.25µg/ml Fungizone
50µg/ml Linoleic Acid BSA
0.04µg/ml Hydrocortisone
0.04µg/ml Thyroid Releasing Hormone (TRH)
9.5µg/ml Insulin
0.002µg/ml Sodium Selenite

To prepare high glucose (HG) medium used for 3D culture, 4.44ml of a 45% D-glucose solution was added to α-MEM Complete Culture Medium to give a final glucose concentration of 25mM.

2.1.3.4 HC-04 Culture Medium

HCM Bullet Kit (Lonza CC-3198); Includes Hepatocyte Basal Medium (HBM) and Singlequots).
10% FCS

2.1.3.5 Caco 2 and LS147T Culture Medium

Phenol Red-Free DMEM (Invitrogen 21063)
10% FCS
100U/ml Penicillin/0.1mg/ml Streptomycin
1.25µg/ml Fungizone
1x Non-essential amino acids
1mM Sodium pyruvate

2.1.3.6 Preparation of FFP

For some studies, human fresh frozen plasma (FFP) was added to culture medium in place of FCS. During collection of FFP, the anticoagulant used was sodium citrate which cannot be used within the LG BAL since it sequesters calcium from alginate destroying polymerisation. In order to prevent this, calcium chloride was added to neutralise the original anticoagulant, and heparin added in its place to prevent clotting.

FFP was obtained from the Royal Free Haematology Department, thawed and pooled from multiple donors according to blood group. 4µl/ml of a 1M $CaCl_2$ solution was added to the thawed FFP along with 40 IU/ml Heparin (Multiparin, CP Pharmaceuticals). 50ml aliquots were stored at -20°C until required.
Prior to media preparation, FFP was thawed overnight at +4°C, centrifuged at this temperature at 1000g for 20 minutes and supernatant then carefully decanted. Culture media containing FFP was passed through a 0.45µM filter prior to use to remove platelets.

2.1.4 Culture of Human Cell Lines

HepG2 cells were originally obtained from the HPACC (ECACC), adapted to long term culture in complete culture medium as described in section 2.1.3.3 and used by the Liver Group (LG) for a number of years. These cells were passaged 1:6 twice a week.

BALG2 cells are early passage HepG2 cells which were obtained from the HPACC in March 2009 and were naïve to any media adaptations. The BALG2 nomination is used throughout this thesis to differentiate from LG HepG2 cells.

HC-04 cells were obtained under licence from Siam Life Science Ltd. (Thailand). These cells were passaged 1:3 every 3-5 days.

Caco 2 cells were from the department of Gastroenterology at the Royal Free Hospital and were passaged 1:6 twice a week.

LS147T cells were kindly provided by Dr Hassan Shahbakhti and Dr Surinder Sharma from the UCL Cancer Institute. Cells were passaged 1:6 twice a week.

Materials

HBSS (without calcium and magnesium)
0.25g/L Trypsin 0.1g/L EDTA (Invitrogen # 15400-054), 1g/L glucose in citrate saline (4.4g/L trisodium citrate.$2H_2O$, 10g/L KCl)
2% Trypan blue in PBS
Tissue culture flasks
Haemocytometer
FCS containing 10% DMSO
2ml Cryovials
Polystyrene container lined with cotton wool
37°C water bath

Method

Cell culture work was performed in a class II microbiological safety cabinet using disposable sterile plastic ware. All cell lines are adherent and were cultured in a 37°C incubator with a humidified atmosphere of 95% air, 5% CO_2. Medium was changed every 2 days and cells were passaged by trypsinisation when 70-80% confluent.

To passage cells, medium was poured from the flasks, cells washed twice with HBSS and then incubated for 1-2 minutes at 37^oC with 1.5ml trypsin-EDTA/$25cm^2$ of growth area. After this time, cells were detached from the surface of the flask by tapping it firmly. An equal volume of warmed medium containing FCS was added to stop the action of the trypsin and cells were centrifuged at 300g (1200rpm) at room temperature for 4 minutes. Supernatant was discarded and the cells resuspended in 5mls growth

medium. The cells were then syringed with a 21G needle 4-5 times to disaggregate cell clumps and flasks seeded at the ratios indicated above.

2.1.4.1 Determination of Cell Number and Viability

Cell number and viability was determined by trypan blue exclusion of non viable cells. This was done by mixing 160µl of HBSS, 20µl of trypan blue and 20µl of homogenous cell suspension. After 2 minutes 10µl of the solution was loaded onto a haemocytometer chamber and viable and non viable cells were counted.

2.1.4.2 Cryopreservation and Reconstitution of Cell Lines

Cell lines were maintained in culture for a maximum of 10 continuous passages, it was therefore necessary to prepare cell bank stocks of each cell line.

Cryopreservation

Following trypsinisation, cells were resuspended in culture medium at a density of $1x10^7$cells/ml. An equal volume of freezing mix (FCS + 10% DMSO) was then added and 1ml aliquots transferred to cryovials. Vials were placed in a polystyrene container lined with cotton wool which was placed at -80°C which allowed freezing to occur at a rate of approximately 1°C/minute. Once frozen, cells were transferred to liquid nitrogen for long term storage.

Cell Revival

To revive cells, cryovials were removed from liquid nitrogen and quickly thawed by swirling in a 37°C water bath. The thawed cell mix was immediately transferred to a 50ml centrifuge tube and 10mls of medium added over a time period of 5 minutes to gradually dilute the freezing mix. Following centrifugation at 300g for 4 minutes at room temperature, supernatant was discarded, cells resuspended in 15mls of medium and then transferred to a T80 flask. Following attachment, medium was replenished and cells maintained in monolayer culture as described above.

2.2 Alginate Encapsulation and 3D Culture

2.2.1 Inotech Cell Encapsulation

Inotech encapsulation was used when small volumes of beads were required for static culture of cells. The Inotech IER-20 Cell Encapsulator system (Inotech, Dottikon, Switzerland) is based on the principle that a laminar liquid jet is broken into equally sized droplets by a superimposed vibration. Additionally, an electrostatic dispersion unit is used to charge the surface of the liquid which transforms the one-dimensional droplet chain into a funnel-like multilane stream. This prevents beads from hitting each other in flight, and from hitting each other as they enter the polymerisation solution (Figure 2-1). When the alginate drops contact the calcium of the polymerisation buffer beads are formed by cross linkage of alginate chains with divalent cations.

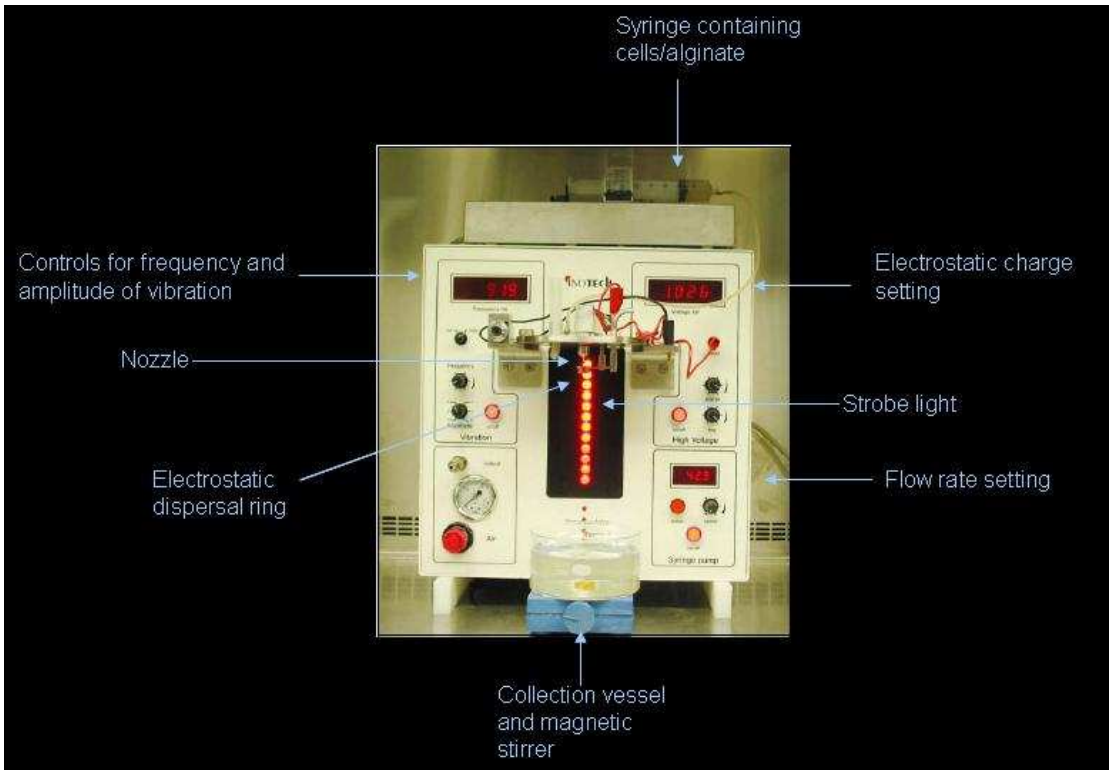


Figure 2-1. Inotech IER-20 Cell Encapsulator System.

Materials

100ml 2% Alginate – pH 7.4

0.15M Sodium chloride

1M Sodium hydroxide

15mM HEPES buffer

2% w/v Alginic acid sodium salt from

Macrocystispyrifera kelp

(Sigma A2033)

Polymerisation Buffer – pH 7.4

0.15M Sodium chloride

1M Sodium hydroxide

15mM HEPES buffer

0.204M Calcium chloride

Consumables

Volumetric flasks

Schott bottle

Magnetic flea

Syringe filter

Baked Pyrex beakers (3 hours at 180°C)

Stainless steel forceps

Inotech syringe pump system (Inotech,

Dottiken, Switzerland)

Bottomless beaker

Elastic band

Nylon mesh

DMEM supplemented with 10% FCS,

P/S and Fungizone

High glucose (HG) medium

Preparation of 2% Alginate Solution

100ml of HEPES buffered saline solution was prepared; pH was adjusted to 7.4 using 1M NaOH. This solution was placed in a foil covered 250ml Schott bottle containing a magnetic stirrer. Alginic acid was added gradually and the solution stirred overnight at a slow speed. The following day it was autoclaved at 121°C for 10 minutes and then left to cool to room temperature before use.

Preparation of Polymerisation Buffer

A 0.204M $CaCl_2$ solution was prepared in HEPES buffered saline. This was pH adjusted to 7.4 using 1M NaOH, autoclaved at 121°C for 25 minutes and then left to cool to room temperature. Pluronic acid was filter sterilised and added immediately before use.

Preparation of Alginate Cell Suspension

Cells were trypsinised, the pellet resuspended in 5ml of HG medium and cell number and viability determined by trypan blue exclusion. Cells were then diluted to the required density (typically $1x10^6$/ml for HepG2 cells) in a mixture of one part 2% alginate to one part culture medium. This solution was drawn up through a 50μM filter into a 50ml syringe and attached to the syringe pump of the Inotech.

Cell Encapsulation and Bead Collection

The cell suspension was passed through the encapsulator at a flow rate of 5ml/min using a 200μM nozzle vibrating at 1295 Hz (vibration amplitude 3), which yielded spherical beads approximately 400μM in diameter. Electrostatic charge was set to 0.5 volts. The flow of beads fell into gently stirred polymerisation buffer where they were maintained for 15 minutes. After this time beads were removed from polymerisation buffer by pouring them into a beaker with a 200μM mesh bottom where they remained whilst being washed twice with DMEM. They were then transferred to 50ml centrifuge tubes and resuspended with HG media. Once beads had settled, the volume collected was estimated from the graduations marked on the side of the tube. Beads were subsequently diluted and cultured as required.

Static Culture of Beads

Beads were resuspended in a $175cm^2$ tissue culture flask with HG media at a ratio of 0.25ml beads to 8ml culture medium. 8mls of this suspension was transferred into each

well of a six well plate. Each well contained a 100μM cell strainer on which the beads rested. This allowed beads to be easily removed from wells during media changes or for assays. Media was replenished every other day.

2.2.2 JetCutter Cell Encapsulation

JetCutter encapsulation was used when large volumes of beads were required. This machinery was operated by more experienced colleagues.

The JetCutter system (geniaLab®) utilises a cutting tool with asymmetrically arranged wires, which is inclined relative to a vertical solid stream of liquid, resulting in a horizontal cut as the tool passes through the liquid stream. This produces cylindrical segments which form beads due to surface tension on their way to a collecting bath which will polymerise the material (Figure 2-2).

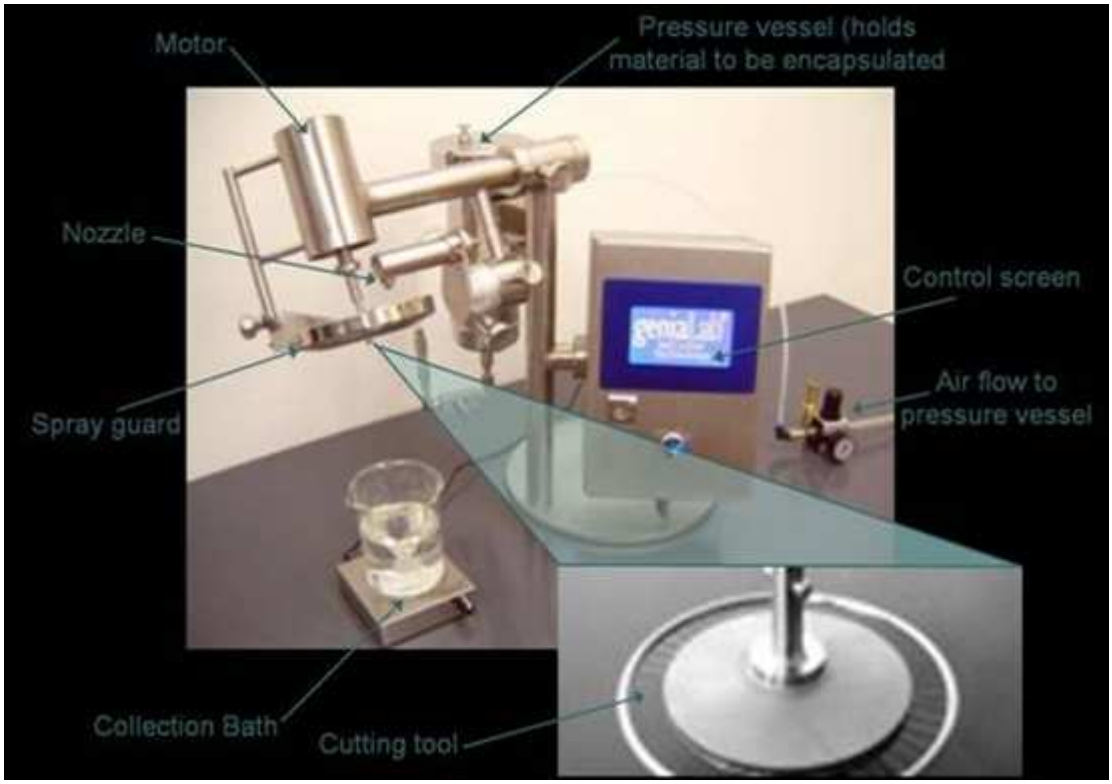


Figure 2-2. Jetcutter encapsulation system.

Materials

2% alginate solution

Polymerisation buffer

HG Culture Media

DMEM medium supplemented with 10% FCS, P/S and Fungizone

Baked Pyrex beakers

Autoclaved JetCutter components (pressure vessel, inline filter, nozzle holder and nozzle assembled and autoclaved, cutting tool, cutting disc, collection cover)

Autoclaved 5L stainless steel basin

Baked funnel for filling pressure vessel

Plastic beaker for collecting waste from JetCutter

25ml measuring cylinder for calibration of flow rate

Plastic bottomless beaker and elastic band

200μm nylon mesh

Magnetic Stirrers

Stainless steel spatula

Sterile consumables including, 15ml Nunc tubes, 50ml Nunc tubes, 50ml syringes, 25ml serological pipettes, Gilson tips and T175 culture flasks

JetCutter Set Up

Cells were prepared in a mixture of one part 2% alginate to one part HG culture medium as described for section 2.2.1. This mixture was added, through a funnel, to the pressure vessel which was immediately capped and the stirring motor set to 50rpm. Air pressure was applied and the pressure vessel outlet tap opened to begin encapsulator flow through. Flow rate was calibrated to 0.33ml/sec by adjusting air pressure. Run parameters on the control panel were set as follows: flow rate 0.33ml/sec, 350μM nozzle size, 60 wires (wire diameter 100), motor speed 3600rpm. JetCutter software was used to calculate optimal angle of inclination of the cutting tool which was then attached along with the cutting disk and collection cover. A 5L basin containing 3L of polymerisation buffer was placed on a magnetic stirrer under the nozzle and set at a low speed.

Cell Encapsulation and Bead Collection

Once setup was completed, the outlet tap on the pressure vessel was opened to commence liquid flow and the Jetcutter motor started. Initial flow through was collected in a beaker and discarded. Once a clean flow through was established, beads were collected in polymerisation buffer. Timing the duration of collection allowed the volume of liquid encapsulated to be calculated. Beads were then poured from their collection basin into a 1L beaker and polymerisation buffer removed by transferring them to a beaker with a 200μM mesh bottom. Beads were retained on the mesh bottom whilst being washed with DMEM and then transferred into 50ml centrifuge tubes where they were resuspended in HG medium. Once beads had settled, the volume collected was estimated from the graduations marked on the side of the tube. Beads were then diluted and cultured as required.

2.2.3 Removing Cells from Alginate

Cell spheroids were removed from alginate by the addition of EDTA which chelates the calcium from the alginate allowing it to dissolve and release the spheroids. Solutions used in this procedure were treated first with DEPC (described in section 2.4.1) to inactivate any RNase enzymes that may be present which could potentially degrade any RNA prepared from spheroids.

Materials

1x sterile PBS
16mM EDTA in 0.15M NaCl treated with 0.1% DEPC, pH7.4
0.15M NaCl treated with 0.1% DEPC, pH7.4
6-well plates
Small spatula
2ml microfuge tubes
2ml syringe
21G needles

Method for Beads Maintained in 6 Well Plates

To harvest cells from beads, they were removed from the wells in their cell strainers and washed twice by immersion into 5ml of cold 1xPBS that had been added to a 6 well plate. The plate was kept on ice throughout the procedure. Using a small spatula the beads were scraped out of the strainers into 2ml microfuge tubes containing 1.8ml

16mM EDTA in 0.15M NaCl. Beads were incubated at room temperature with this solution for 15 minutes and then syringed 4-5 times using a 21g needle to disaggregate the cell spheroids. The samples were then centrifuged at 5000g for 5 minutes at 4°C, the supernatant removed and the pellet washed with 1.5ml of 0.15M NaCl and centrifuged again. Remaining supernatant was removed leaving the cell pellet to be used for subsequent experiments.

Method for Large Volumes of Beads Maintained in Bioreactor Culture

Beads were distributed evenly into 50ml centrifuge tubes (approximately 10ml volumes) and 35mls of 16mM EDTA in 0.15M NaCl added. The solution was incubated at room temperature for 15 minutes with regular mixing by inversion and then centrifuged at 1500g for 15 minutes. The pellet was washed with 10ml of 0.15M NaCl and centrifuged again. Remaining supernatant was then removed leaving the remaining cell pellet.

2.3 Analysis of Cell Viability and Protein Content

2.3.1 FDA/PI Staining

Fluorescein diacetate (FDA) is cell-permeable and is hydrolysed by esterases that are only present in metabolically active cells to form a fluorescent compound. Propidium iodide (PI) is a membrane impermeant dye which emits a red fluorescence when bound to DNA. This occurs only when cell membranes are compromised and represents non-viable cells [218].

Materials

1mg/ml FDA in DMSO
1mg/ml PI in water
1x PBS containing calcium and magnesium
1ml pipette with the end cut down by ~ 2mm
Microscope slides
Coverslips
Nikon Eclipse microscope
DX1200 camera
FDA filter block (excitation filter of 465-495nm, emission filter of 515-555nm)
PI filter block (excitation filter of 510-560nm, emission filter of 590nm)

Method

A sample of alginate beads was transferred in culture medium to a 1.5ml microfuge tube, allowed to settle and the medium aspirated. Beads were washed twice with PBS and resuspended in 500µl of PBS. 20µl of PI and 10µl of FDA were added, the solution gently mixed with the beads and allowed to stand for 90 seconds. The beads were then washed once more with PBS and finally resuspended in 500µl of PBS. A portion of the stained beads were transferred to a microscope slide using a cut down pipette tip to allow the beads to pass through without damage. Excess liquid was removed with a tissue and a coverslip placed over the beads. The beads were then visualised with a fluorescence microscope. Phase contrast, live and dead images were captured using Lucia Imaging Software with a DX1200 camera. Live and dead images were normally taken at x10 magnification at exposures of 32ms and 250ms respectively.

2.3.2 Tetrazolium Salt Viability Assays

Tetrazolium salts are reduced to a formazan product by mitochondrial reductase enzymes in active cells. This conversion can therefore be directly related to metabolically active cells which can be quantified by determining increase in absorbance readings, relating to increases in product formation. WST-1 was selected for use in this project since, following exposure, cells remain intact and so can be used for further studies.

Materials

2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1-NBS Bioligicals W201)
1-Methoxy-5-methyl-phenazinium (mPMS)
Serum free culture media
HBSS
96 well clear flat bottomed culture plate
Spectrophotometer at 450nm

Method

A solution of prewarmed culture media containing 500µM WST-1 and 100µM mPMS was freshly prepared immediately prior to each assay. Cells were washed twice with

HBSS and an appropriate volume of reaction mix added to each well of cells (1ml for a 12 well plate or 100µl for a 96 well plate) which were incubated in a humidified 37°C 5% CO_2 incubator for 1 hour. After this time 100µl of culture supernatant was transferred to a 96 well plate and absorbance read at 450nm.

2.3.3 Bicinchoninic Acid Protein Assay

The Bicinchoninic Acid (BCA) assay was used to determine protein content of samples. Presence of protein resulted in a colour change from green to purple which was quantified by determining increases in absorbance readings which relate directly to increases in protein concentration [219].

Materials

BCA Reagent: 1% BCA, 2%Na_2CO_3, 0.16% NaK tartrate, 0.4% NaOH, 0.95% $NaHCO_3$ - pH corrected to 11.25 using NaOH
4% Copper Sulphate
10mg/ml Bovine Serum Albumin (BSA)
Plate sealer
Plate shaker
96 well clear flat bottomed culture plate
Spectrophotometer at 570nm

Method

A standard curve was prepared using BSA in PBS (1-0.03 mg/ml). Immediately prior to the assay, BCA reagent and copper sulphate were mixed at a ratio of 50:1. 100µl of this reagent was added to each well of a 96 well plate, along with 100µl of standard or sample. The plate was sealed, mixed on a plate shaker for 30 seconds and then incubated at 37°C for 30 minutes. Absorbance was read using a spectrophotometer at 570nm.

2.4 Molecular Biology Methods

2.4.1 DEPC Treatment of Solutions

Diethylpyrocarbonate (DEPC) was used to treat laboratory prepared solutions to remove RNase enzymes (although it is not compatible with Tris buffers or HEPES). Solutions were treated overnight with 0.1% v/v DEPC (Sigma D5758) and then autoclaved for 25 minutes at 121°C to inactivate the DEPC yielding CO_2, H_2O and ethanol.

2.4.2 DNA Precipitation by Ethanol/Sodium Acetate

Ethanol precipitation was used to purify and concentrate DNA. Glycogen acts as an inert carrier to allow visualisation of low concentrations of DNA. This procedure was carried out on ice. All centrifugation steps were at 4°C.

Materials

100% Ethanol

3M Sodium Acetate (pH 5.2)

20mg/ml Glycogen from mussels (Roche)

70% Ethanol in DEPC treated sterile H_2O

Method

DNA to be precipitated was measured into a sterile microfuge tube along with 1µl of glycogen. A $1/10^{th}$ volume of sodium acetate was added and the solution mixed by vortexing. A 2.5 x volume of chilled ethanol was then added, mixed by vortexing and incubated on ice for 1 hour. After this time, tubes were centrifuged at 13 000g for 15 minutes and the supernatant discarded. The remaining pellet was washed with 200µl of chilled 70% ethanol and centrifuged for a further 10 minutes. The pellet was air dried, redissolved in 6µl of sterile water and quantified using a NanoDrop at 230nm.

2.4.3 RNA Extraction and DNase Treatment

RNA extraction and precipitation was performed according to the manufacturer's instructions. With the exception of 75% ethanol, all reagents were supplied with the RNA isolation kit. All procedures were carried out on ice, all centrifugation steps were performed at +4°C and all consumables were certified DNase and RNase free.

Following isolation, RNA was treated with DNase to remove any trace amounts of contaminating genomic DNA which may interfere with subsequent reactions.

Materials

RNAgents Total RNA Isolation System (Promega Z5110)
75% Ethanol in DEPC treated sterile H_2O chilled to -20°C
RQ1 RNase-Free DNase (Promega M6101)
Sterile 2ml microfuge tubes
Sterile 0.2ml PCR tubes
ND1000 Spectrophotometer (NanoDrop Technologies)
GeneAmp® 2700 thermocycler (Applied Biosystems)

RNA Isolation

To prepare RNA from freshly isolated primary human hepatocytes and HepG2 spheroids, $1x10^6$ cells were transferred to a sterile microfuge tube and pelleted by centrifugation at 1000g. Supernatant was aspirated and cells resuspended in 120µl denaturing solution. For monolayer cultures, media was aspirated and 120µl (12 well plates) or 60µl (96 well plates) of denaturing solution added. The mixture was pipetted several times to disrupt the cells and transferred to a sterile microfuge tube.

To the disrupted cell suspension, a $1/10^{th}$ volume of 2M Sodium Acetate was added and mixed by inversion, followed by an equal volume of Phenol:Chloroform:Isoamyl Alcohol. The tube was capped and the contents mixed by inversion, shaken vigorously for 10 seconds and then chilled on ice for 15 minutes. After this time the tubes were centrifuged at 10,000g for 20 minutes. The top aqueous phase was transferred to a fresh microfuge tube, taking care not to disturb the interphase material and an equal volume of isopropanol added. RNA was precipitated at -20°C for 30 minutes and then pelleted by centrifugation for 10 minutes at 10,000g. The supernatant was decanted and 1ml of chilled 75% ethanol added to wash the RNA which was then pelleted once more by centrifugation. The supernatant was removed and the RNA was air dried for 5-15 minutes and resuspended in 15µl of nuclease-free water.

RNA was quantified by spectrophotometry at 230nm using the NanoDrop and purity assessed from the associated A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios. 1µg of RNA was retained for DNase treatment and the remainder was stored at -80°C.

DNAse Treatment

To treat RNA with DNase, the following reaction was prepared in a 0.2ml PCR tube:

1µg RNA
1µl 10x RQ1 buffer
1unit (1µl) RQ1 DNase
Nuclease free H_2O to a final volume of 10µl

The reaction tubes were incubated at 37°C for 30 minutes. After this time 1µl of RQ1 DNase stop solution was added to the tubes which were heated to 65°C for 10 minutes to inactivate the DNase and then cooled to 4°C.

2.4.4 cDNA Synthesis

DNase treated RNA
RevertAid™ First Strand cDNA Synthesis Kit (Fermentas K1622)
0.2ml PCR tubes
GeneAmp® 2700 thermocycler (Applied Biosystems)

cDNA synthesis was carried out according to manufacturer's directions. The tubes containing the DNase treated RNA (section 2.4.2) were centrifuged for 5 seconds at 5000g to collect the contents and 1µl of random hexamer primers added. This was mixed gently, incubated at 70°C for 5minutes, chilled on ice and centrifuged for 5 seconds. The tubes were then returned to ice and to each tube the following was added in order: 4µl 5x reaction buffer, 1µl RiboLockTM Ribonuclease Inhibitor and 2µl 10mM dNTP mix. The tubes were incubated at 25°C for 5 minutes and then 1µl RevertAid™ M-MuLV Reverse Transcriptase added. The tubes were incubated at 25°C for 10 minutes, 42°C for 60 minutes and finally 70°C for 10 minutes (to stop the reaction) before being chilled on ice. cDNA was stored at -20°C until required.

2.4.5 Reverse Transcriptase –Polymerase Chain Reaction (RT-PCR)

2.4.5.1 Preparation of Olignucleotides

Oligonucleotide primers were selected that had been validated by others in quantitative RT-PCR assays. Sequences are given in Table 2-1. Lyophilised primers, which were custom synthesised by SigmaGenosys, were solubilised to 100µM in sterile H_2O. These stocks were diluted 1:10 to provide a 10µM working stock solution. All primer solutions were stored at -20°C.

Table 2-1. Primers used for the analysis of gene expression by RT-PCR listed in 5'-3' orientation

Gene		Primer Sequence	Reference
CYP1A2	Forward	TGTTCAAGCACAGCAAGAAGG	220
	Reverse	TGCTCCAAAGACGTCATTGAC	
CYP3A4	Forward	GATTGACTCTCAGAATTCAAAAGAAACTGA	220
	Reverse	GGTGAGTGGCCAGTTCATACATAATG	
CYP3A7	Forward	CCTTACCCCAATTCTTGAAGCA	220
	Reverse	TCCAGATCAGACAGAGCTTTGTG	
CAR	Forward	GCAAGGGTTTCTTCAGGAGAAC	221
	Reverse	CTTCACAGCTTCCAGCAAAGG	
GR	Forward	GAGGAGGAGCTACTGTGAAGGTTT	221
	Reverse	GCTGCTTGGAGTCTGATTGAGA	
PXR	Forward	GGCCACTGGCTATCACTTCAA	221
	Reverse	TTTCATGGCCCTCCTGAAAA	
RXRα	Forward	GAGACCTACGTGGAGGCAAA	67
	Reverse	GATGGAGCGGTGGGAGA	
VDR	Forward	TAGAGCTGTCCCAGCTCTCCAT	221
	Reverse	TGACCTTTTGGATGCTGTAACTGA	
18s	Forward	CTTAGAGGGACAAGTGGCG	13
	Reverse	GGACATCTAAGGGCATCACA	

2.4.5.2 RT-PCR Set-up and Cycling Conditions

Materials

HotStarTaq Polymerase Master Mix (HST, Qiagen 1039620)

Custom Synthesised Oligonucleotides (SigmaGenosys) diluted to 10µM (final reaction concentration = 500nM)

Sterile (RNase DNase free) H_2O (Qiagen 130181929)

GeneAmp® 2700 thermocycler (Applied Biosystems)

0.2ml PCR Tubes

Method

Each PCR reaction contained the following:

Reagent	**µl/20µl Reaction**
2x HST	10
Forward Primer	1
Reverse Primer	1
H_20	7
cDNA	1

For each set of primers, a mastermix was prepared and 19µl of this was aliquoted into 0.2ml PCR tubes. 1µl of cDNA or H_2O for no template control (ntc) was added and PCR performed in a GeneAmp® 2700 thermocycler.
HotStarTaq was activated by heating at 95°C for 15 minutes. Cycling conditions were then 15s at 94°C for denaturation, 30s at primer specific annealing temperature and 40s for extension at 72°C. For each primer, cycle number and annealing temperatures are shown in Table 2-2.

Table 2-2. Annealing temperature, number of cycles and expected fragment size for each primer set used in this thesis

Gene	Annealing Temp (˚C)	Cycles	Fragment Size (base pair)
CYP1A2	60	55	91
CYP3A4	62	45	148
CYP3A7	61	55	197
CAR	61	55	72
GR	61	55	75
PXR	62	55	70
RXRα	59	55	199
VDR	61	55	69
18s	60	35	70

2.4.5.3 Agarose Gel Electrophoresis

The density of the agarose gel used depended upon the size of fragments that were separated. To separate 500 bp-3 kb fragments a 1% agarose gel was used. To separate fragments < 500 bp a 2% agarose gel was used.

Materials

Agarose (Bioline BIO-41025)

50xTAE stock (For 1L in ddH_2O: 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA in ddH_20, pH 8.0)

Ethidium Bromide 10mg/ml stock (stored at 4°C in the dark)

Hyperladder I and IV (Bioline)

5x Loading Buffer (Bioline BIO-37045)

Gel electrophoresis tank (BioRad)

Microwave

Method

0.5g (1% gel) or 1g (2%) of agarose was dissolved in 50ml 1xTAE using a microwave. When the agarose had cooled to ~60°C, 1µl ethidium bromide was added and the gel poured into the casting tray and left to solidify. The gel was immersed into a gel electrophoresis tank filled with 1xTAE and the wells of the gel flushed gently with TAE. 5µl DNA Hyperladder I or IV was used for reference (Figure 2-3). PCR samples were mixed with 5x loading dye (1x final) and loaded into the wells. The gel was run for 45 minutes at 90V 140 Amps at constant voltage.

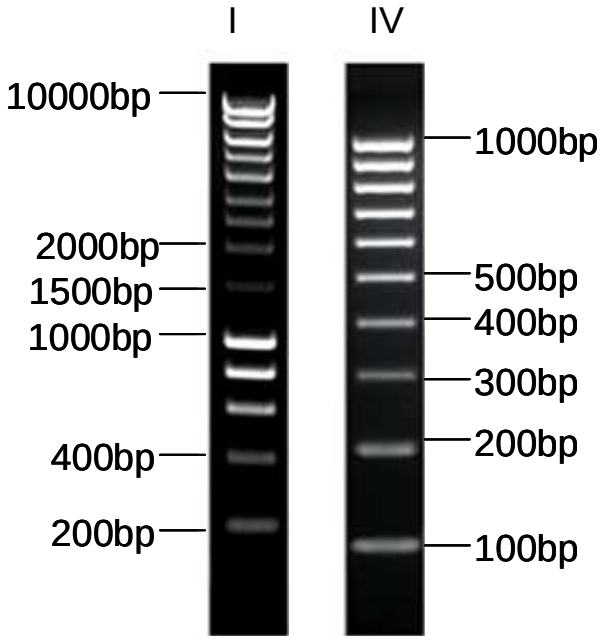


Figure 2-3. DNA ladders were obtained from Bioline. (left) Hyperladder I was used for 1% agarose gels and (right) Hyperladder IV was used for 2% agarose gels or DNA fragments up to 1000 bp.

2.4.5.4 Reverse Transcriptase Quantitative PCR (RT-qPCR)

In traditional PCR, results are obtained as an end-point, during the plateau phase, when the reaction has run to completion. Quantitative PCR allows the reaction to be monitored as it progresses through the exponential phase, and is achieved by incorporation of a fluorescent reporter. At the end of the PCR run a threshold is defined which is a function of the background fluorescence. For each reaction in the run, it is possible to determine the cycle number at which this threshold is crossed, termed the Cycle threshold (C_t) which is proportional to the amount of starting template. Copy number of the gene of interest can be calculated by generating a standard curve and normalisation to a reference gene or to ribosomal RNA allows the relative quantity of gene expression to be calculated. This thesis uses Sybr Green I which binds the minor groove of double stranded DNA molecules and emits a fluorescent signal when bound

but exhibits a low amount of fluorescent signal when in solution. A disadvantage of Sybr Green is that non-specific PCR products and primer dimer complexes will also contribute to the fluorescent signal affecting product specificity. This can be avoided by PCR optimisation and melt curve analysis in which the products of the PCR are separated by gradually increasing the thermocycler from the annealing to the denaturing temperature following the end of the PCR (Figure 2-4). This is equivalent to running the PCR products on a gel, where the only visible band should correspond to the amplified product.

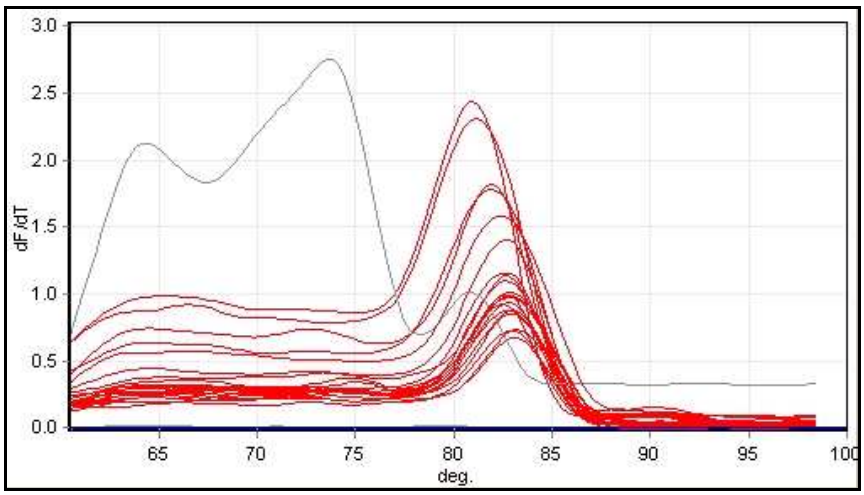


Figure 2-4. An example of melt curve analysis which is achieved by gradually increasing the temperature of the thermocycler. SYBR green dye is released into the reaction mix as the amplicon denatures and this occurs at a specific temperature for each product formed. Overlapping peaks shown in red represent primer specific products, the lone grey peak is an example of non-specific product formation.

Materials

HotStarTaq Polymerase Master Mix (HST, Qiagen 1039620)

Custom Synthesised Oligonucleotides (SigmaGenosys) diluted to 10µM (final reaction concentration = 500nM)

Sterile (RNase DNase free) H_2O (Qiagen 130181929)

Hyperladder I and IV (Bioline)

5x Loading Buffer (Bioline BIO-37045)

Montage DNA extraction columns (Millipore LSKGEL050)

NanoDrop Spectrophotometer

Sybr Green I (Invitrogen S7567) 1:10 000 dilution of original stock in 0.01% DMSO (1:200 000 dilution in final reaction)

0.2ml PCR tubes

Rotorgene RG-3000 Cycler (Corbett Life Science)

Preparation of Standards

In order to produce standard curves to quantify gene expression, standards of known copy number were generated. PCR was carried out, in triplicate, on human liver cDNA using each set of primers as described above. Product was run on a 2% agarose gel, bands visualised using a UV light box and then quickly excised, transferred into a sterile microfuge tube and frozen at -20°C for 1 hour. The gel was thawed on ice and transferred to a Montage DNA Gel Extraction Column (Millipore) and DNA extracted by centrifugation at 5000g for 10 minutes. DNA was concentrated by ethanol precipitation (section 2.4.2), resuspended in 10µl sterile H2O and quantified using the NanoDrop. Using the concentration of DNA and the fragment length created by the primers, the (estimated) number of molecules per µl of cDNA was then calculated as follows:

Average weight of a base pair (660) x amplicon length = Molecular weight (MW) of amplicon

MWg (number of atoms in one mole) = Avogadros number 6.022×10^{23}

Amplicon molecules/ng $= 6.022 \times 10^{14}$ */amplicon MW*

Amplicon molecules (copy number)/µl= concentration (ng/µl) x amplicon molecules/ng

Standard concentration was adjusted to 10^{10} copies/µl using sterile H_20 and then standards decreasing in 10-fold increments (10^{10}-10^{1} copies/µl) were prepared and stored at -20°C as 5µl single-use aliquots.

RT-qPCR

Each PCR reaction contained the following:

Reagent	**µl/20µl Reaction**
2x HST	10
Forward Primer	1
Reverse Primer	1
Sybr Green I	1
H_20	6
cDNA	1

For each set of primers, a mastermix was prepared and 19µl of this was aliquoted into 0.2ml PCR tubes. 1µl of cDNA, standard or H_2O no template control (ntc) was then added and PCR performed in a Rotorgene RG-3000 thermocycler using the conditions described in section 2.4.5.2. Copy number was calculated using a standard curve and for each gene, expression levels were normalised to ribosomal 18s RNA.

2.5 Analysis of Cytochrome P450 Function and Induction

2.5.1 Cytochrome P450 Inducers

Dexamethasone (Sigma D4902)
Dibenz [a,h] anthracene (Sigma D8543)
Indirubin (Biomol CC206-0005)
Phenobarbital (Sigma P5178)
Rifampicin (Sigma R3501)
1α, 25-dihydroxyvitamin D_3 (VD3 – Sigma D1530)
DMSO (Sigma spectrophotometric grade 154938)

1000x stock solutions of inducers were prepared in DMSO (inducer concentrations will be indicated in subsequent chapters). These solutions were prepared and stored in light protective 2ml ampoules. Solutions were kept at room temperature and fresh stocks prepared every 12 weeks. Inducers were added to culture media by sequential dilution (1:10 then 1:100) to give a final DMSO concentration of 0.1% (v/v). A 0.1% DMSO vehicle control was included in each experiment.

2.5.2 Functional Assays

Assays for cells in monolayer culture were performed in microplate format. Assays using freshly isolated primary human hepatocytes in suspension, or encapsulated cells, were performed in 1.5ml microfuge tubes.

For each assay, a standard curve (in duplicate) was included along with controls of cells without substrate and substrate without cells. Data was background corrected and then adjusted for protein content during analysis.

2.5.2.1 Ethoxycoumarin Assay to Determine Broad Spectrum CYP Activity

7-Ethoxycoumarin (7-EC) was used as a substrate to determine general P450 activity since 7-Ethoycoumarin *O*-deethylation (ECOD) resulting in 7-Hydroxycoumarin (7-HC) product formation may be catalyzed by CYP enzymes from CYP1, CYP2 and CYP3 families [214].

Materials

7-Ethoxycoumarin (7-EC, Sigma E1379, 40mM in MeOH)
Serum free culture media
HBSS
7-Hydroxycoumarin (7-HC, Sigma H2400, 160mM in 1M NaOH)
Chloroform
0.01M NaOH/1M NaCl in deionised H_2O
1.5ml Microfuge tubes
Microfuge
Vortex
96 Well clear flat bottomed plate
Cytofluor 4000 fluorescence reader (PerSeptive Biosystems)

Method

7-EC was diluted in pre-warmed culture media to give a final concentration of 200µM. Cells were washed twice with HBSS and an appropriate volume of 200µM 7-EC added to the culture (500µl or 100µl for 12 well or 96 well plates respectively). Plates were incubated at 37°C for one hour and then the supernatant was transferred to a 1.5ml microfuge tube. A standard curve of 7-HC (50nm -1.5nm + no standard blank control) was prepared in culture medium containing 200µM EC. Product was extracted by adding 900µl of chloroform, mixing well and then vortexing. Tubes were centrifuged at 3000g for 1 minute and the product back extracted by transferring 500µl of the lower organic phase to a 1.5ml microfuge tube containing 500µl of 0.01M NaOH/1M NaCl. This mixture was vortexed and then centrifuged at 3000g for 1 minute. 200µl of the upper aqueous phase was then transferred to a 96 well plate and read using a Cytofluor (Excitation 364/40, Emission 460/40). Cells were harvested for protein by washing twice with PBS and adding PBS 0.5% Triton X. Plates were then frozen until analysis using the BCA assay. Data was normalised for total protein content during analysis.

For encapsulated cells, 0.25ml of beads were incubated with 3ml of substrate.

2.5.2.2 Promega P450 Glo Assays to Determine Isoform Specific CYP Activity

Materials

Phenol red-free culture media without serum

Promega P450-Glo CYP1A Assay (Luciferin ME V8772)

Promega P450 Glo CYP3A Assay (Luciferin BE V8802)

Sulfaphenazole (Sigma S0758, 10mM MeOH)

d-Luciferin (from beetles, Promega E160, 2mM in H_2O)

White flat bottomed 96 well plates

Tropix TR717 Microplate Luminometer (Applied Biosciences)

Method

In whole cells, assays were performed according to the manufacturer's directions. Briefly 50µM Luciferin-ME +/- 10µM sulfaphenazole or Luciferin-BE was added to prewarmed culture media. Cells were washed twice with unsupplemented media and an appropriate volume of substrate added to cells, either 500µl for a 12 well plate, 200µl for a 48 well plate or 25µl for a 96 well plate. Plates were incubated at 37°C for 1 hour. Supernatant was removed from cells and cells harvested for protein as above. 25µl of supernatant was then transferred to white opaque 96 well plates and an equal volume of manufacturer's luciferin detection reagent added. Plates were incubated in the dark for 20 minutes and then read using a luminometer. A d-luciferin standard curve was included on each plate (2.5 – 0.04 nm). Data was normalised for total protein content during analysis.

For encapsulated cells, 25µl of beads were incubated with 100µl of substrate.

2.5.2.3 Invitrogen Vivid® BOMFC Assay

Vivid ® BOMFC (benzyloxymethylfluorescein) is metabolised by CYP3A and CYP2B6. Substrate metabolism was therefore measured in the presence or absence of the CYP3A inhibitor clarithromycin in order to differentiate between CYP3A and CYP2B6 function.

Materials

Phenol red free culture media without serum

Vivid ® BOMFC Substrate (Invitrogen P2976, 2mM in Acetonitrile)

Vivid ® Cyan Fluorescent Standard (7-hydroxy-4-trifluoromethylcoumarin, Invitrogen P2877, 100µM in DMSO)

Clarithromycin (Sigma C9742, 100mM MeOH)

96 Well clear flat bottomed plate

Cytofluor 4000 fluorescence reader (PerSeptive Biosystems)

Method

BOMFC substrate was diluted in prewarmed culture medium to 2.5µM +/- 100µM clarithromycin. Cells were washed twice with unsupplemented media and an appropriate volume of substrate added to cells, either 500µl for a 12 well plate, 200µl for a 48 well plate or 25µl for a 96 well plate. Plates were incubated at 37°C for 1 hour. Supernatant was removed from cells and cells harvested for protein as above; 25µl of supernatant was then transferred to a clear bottomed 96 well plate which was read using a Cytofluor (Excitation 450/50, Emission 530/25). A Cyan standard curve was included on each plate (250µM-150nm). Data was normalised for total protein content during analysis.

For encapsulated cells, 25µl of beads was incubated with 100µl of substrate.

2.5.2.4 HPLC Analysis of CYP Mediated Testosterone Depletion and Metabolite Formation

Separation of testosterone and its metabolites was performed by HPLC. Subsequent quantification of testosterone depletion was used as a measure of general CYP3A activity whereas quantification of the formation of one of its metabolites, 6β-hydroxytestosterone, was used as a specific measure of CYP3A4 activity.

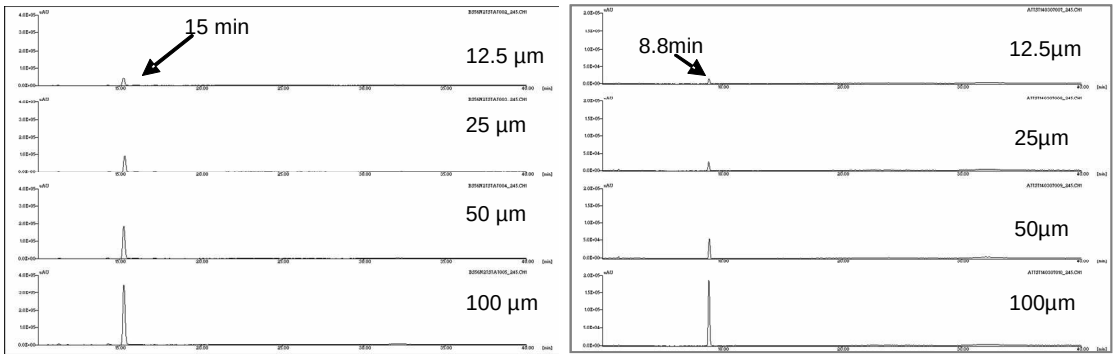


Figure 2-5. Standard curves to demonstrate separation of testosterone at 15 minutes and 6β-hydroxytestosterone at 9minutes. Concentrations may be related to peak area using JASCO HPLC software.

Materials

Phenol red free culture media without serum

Testosterone (Sigma T1500, 40mM MeOH)

6β-Hydroxytestosterone (Sigma H2898)

Nova-Pak C_{18} Column, 3.9 x 150mm, 4µM, Waters

Mobile Phase A: 15% Acetonitrile (Far UV grade Sigma 34888) in deionised H_20

Mobile Phase B: 90% Acetonitrile in deionised H_20

JASCO HPLC machine and accompanying software

Method

Testosterone was diluted in prewarmed culture media to give a final concentration of 200µM. Cells were washed twice with unsupplemented media and an appropriate volume of substrate added to cells (1ml for a 12 well plate, 500µl for a 48 well plate or 100µl for a 96 well plate). Plates were incubated at 37°C for 1 hour and supernatant

then removed and immediately frozen and stored at -80°C for subsequent HPLC analysis. Cells were harvested for protein analysis using the BCA method.

For encapsulated cells, 0.25ml of beads was incubated with 3ml of substrate.

All solvents used for HPLC were degassed by sonication for 15 minutes prior to use. Standard samples consisting of assay media containing 200µM-6µM of both testosterone and 6β-OHTST were included in each run. Following centrifugation at 12000g for 5 minutes to remove any residual protein fragments, 100µl of sample was injected onto the column. The mobile phase was run at 1ml/min from initial starting conditions of 70% A:30% B to 30% A:70%B over 20 minutes. It was maintained at this level for a further 5 minutes before returning to initial conditions over 10 minutes. UV detection of testosterone and metabolite were monitored at 247nm at around 15 minutes and 9 minutes respectively. In order to quantify testosterone depletion and 6β-OHTST formation, standard curves of each were constructed by using JASCO software to relate peak area to concentration. Data was then normalised for total protein content during analysis.

2.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.
Comparisons between two values were made using Student's t test (two-tailed).
One-way ANOVA was chosen when comparing more than two values.
Two-way ANOVA was also used to analyse samples differing in two independent conditions, such as cell type and inducer.

For ANOVA, significance is quoted as $F=(degrees\ of\ freedom, sum\ of\ squares)\ X, P<Y$

A Bonferroni post test was applied to ANOVA analysis to account for multiple comparisons when determining P values.

Unless stated otherwise, values in text, tables and figures were expressed as the mean ± standard deviation (SD) and *n* numbers are given in the accompanying text.
Generally, $P<0.05$ was considered to be significant. In Tables and Figures,
P values are expressed as * ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$).

CHAPTER 3 Characterising Cytochrome P450 Activity in Human Liver to Establish a Gold Standard Comparator

The aim of this thesis was to develop a human cell line with Cytochrome P450 function equivalent to that measured in adult human liver. In order to achieve this it was therefore necessary to determine appropriate comparators. A number of approaches were used.

i.) **Primary human hepatocytes (PHH):** CYP activity was measured immediately (<1 hour) post isolation in human hepatocytes obtained from a range of donors. PHH were also maintained in monolayer culture in order to determine culture conditions that may affect CYP activity. Confluency of hepatocyte cultures was considered, as well as duration of culture and presence of growth factors, and serum or plasma, in culture medium.

ii.) **Human liver microsomes (HLM):** CYP activity was measured in HLM isolated from a number of donors to investigate the hypothesis that interindividual variation has a bigger influence on CYP activity than hepatocyte isolation and culture conditions.

iii.) **Human foetal hepatocytes (FHH):** basal and inducible CYP activity was examined in human foetal hepatocytes using a panel of different CYP inducers.

The work described in this Chapter also provided the context for developing and validating the methods selected for use in this thesis.

3.1 Cytochrome P450 Activity in Adult Human Liver

Liver samples were obtained from the Surgical Department at the North Hampshire Hospital, Basingstoke, UK, from patients undergoing surgery for metastases of colon cancer. Patients gave informed consent for the use of resected tissue for research, approved by the Research and Ethics Committee of the hospital. Throughout this thesis each sample has been assigned the prefix PHH followed by a number.

3.1.1 Primary Human Hepatocytes

CYP activity was measured in PHH immediately post isolation. This was done for a number of donors to give an index of interindividual variation. CYP activity was also measured following time in culture. Effects of culture medium components were considered comparing CYP activity in culture medium containing FCS, FFP or no serum; in addition effects of growth factors in culture medium were measured. Finally inducible CYP activity was measured in adult PHH.

3.1.1.1 Methods

3.1.1.1.1 Isolation of Hepatocytes from Adult Human Liver

Chelating Buffer (20mM HEPES, 0.5mM EGTA in PBS)
Perfusion Buffer (20mM HEPES in PBS)
Digestion Buffer (20mM HEPES, 1.5g BSA (0.5% w/v), 50µg/ml ascorbic acid and 4µg/ml insulin in 300ml HBSS with calcium and magnesium. Immediately prior to digestion of the liver 150mg Collagenase Type IV (0.05% w/v) and 30mg DNase I (0.01%w/v) were added)
Dispersal Buffer (50ml (10%v/v) FCS and 50mg DNase I (0.01% w/v) in 500ml Williams' Medium E (WEM))

Primary human hepatocytes were isolated from a freshly resected piece of liver by two step collagenase perfusion and differential centrifugation [206]. This procedure was carried out by other colleagues.

Donors were both male and female and ranged in age from 39-85 years old. Cell viability was determined by trypan blue exclusion and was between 45-80% (Table 3-1). Due to the limited supply of hepatocytes available from each donor liver, it was not possible to use the same hepatocytes for all experiments. The experimental use of each hepatocyte preparation is therefore indicated in Table 3-1.

Table 3-1. Available donor information and % viability of isolated hepatocytes for each liver sample used in this thesis for (a) measurement of CYP function in freshly isolated hepatocytes, (b) HLM preparation, (c) RNA isolation, (d) monolayer culture.

*** Tissue used for RNA isolation only, therefore no hepatocyte viability information was obtained from this donor.**

PHH #	Sex	Age	% Viability	Experimental Use
1	F	63	75	a, c, d
2	M	68	45	a,
3	M	58	57	b, d
4	M	42	73	b, d
5	M	74	65	b, c
6	M	55	77	c
8	M	81	68	b, c, d
9	F	73	80	a, b, c
10	F	53	61	a, b, c, d
11	F	60	50	a
12	M	61	49	a
13	M	60	58	a, c
14	M	78	47	a
15	M	69	68	a
16	F	45	52	a, c
17	M	47	72	a
18	F	76	64	a, d
19	M	85	65	d
22	F	39	n/a*	c
25	F	64	71	b
28	F	58	67	b
29	M	71	74	d
30	F	57	58	c, d
31	F	73	63	a, b, d
32	F	66	61	d, c
33	F	79	47	c

3.1.1.1.2 Determination of CYP Activity in Freshly Isolated Adult Primary Human Hepatocytes

Hepatocytes were resuspended in Adult Hepatocyte Medium (AHM Chapter 2) at a density of $1x10^6$cells/ml and CYP activity measured within an hour of isolation by ECOD activity (1ml assay volume), testosterone metabolism (1ml assay volume) or luciferase assays (100µl assay volume). Luciferin ME metabolism was measured alone and in the presence of 10µM sulfaphenazole to inhibit CYP2C function. During

analysis, data was normalised for protein content which was determined by the BCA assay and then adjusted to account for viable cells only.

3.1.1.1.3 Culture of Adult Primary Human Hepatocytes

Primary cells were maintained in an incubator at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Post isolation, hepatocytes were resuspended at a density of $1x10^6$ cells/ml in AHM, supplemented with either 10% FCS or 10% (human) FFP. 1ml/well was added to collagen coated 12 well plates and cells incubated overnight to allow attachment.

The following morning, cells were washed twice with prewarmed unsupplemented WEM to remove unattached debris and medium replaced with AHM with or without either FCS ± HGF (20ng/ml) and EGF (10ng/ml) or FFP. Medium was replenished daily and CYP activity measured either by ECOD activity, testosterone metabolism or luciferase assays. Protein content was determined by the BCA assay.

3.1.1.1.4 Induction of CYP Activity in Adult Primary Human Hepatocytes

Inducers were added to PHH either 24 or 48 hours post isolation. Cells were treated for up to one week with inducers, which were replenished daily. CYP activity was then quantified by ECOD activity, BOMFC metabolism or luciferase assay.

3.1.1.2 Results

3.1.1.2.1 Interindividual Variation in CYP Activity in Freshly Isolated Primary Human Hepatocytes

CYP function varied considerably when measured in freshly isolated PHH isolated from different donors (Figure 3-1).

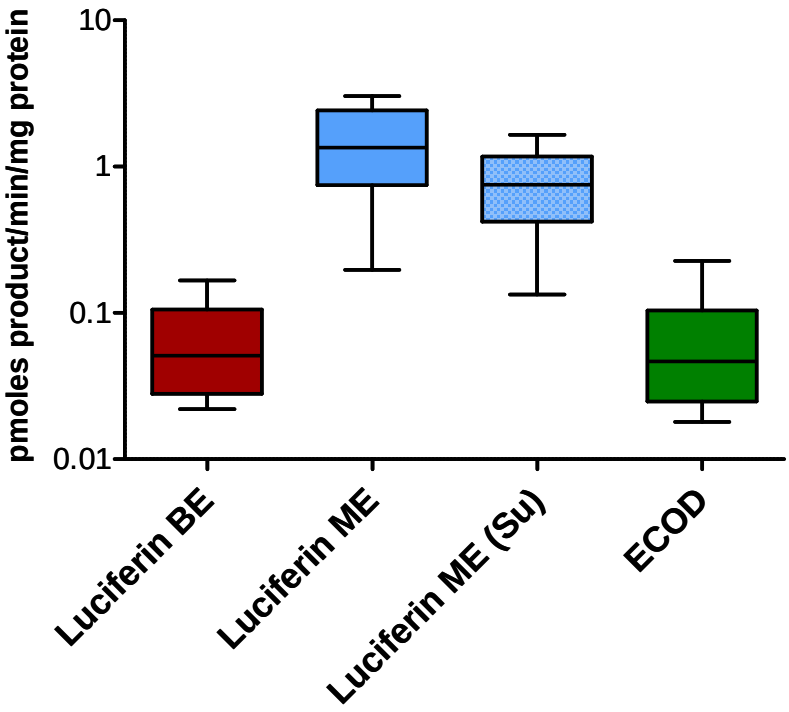


Figure 3-1. Cytochrome P450 function in freshly isolated PHH. For each assay, activity was measured in quadruplicate in each PHH preparation. The average and range of activity measured in PHH isolated from 8-10 separate donors was then calculated and is expressed here for each assay. Note that the *y* axis is plotted on a logarithmic scale. (Su) = measured in the presence of 10µM sulfaphenazole.

The mean activity level and range of function for each assay performed in freshly isolated PHH are summarised in Table 3-2.

Table 3-2. Mean, median and range values (pmoles product/min/mg protein) for Cytochrome P450 function in freshly isolated PHH from 8-10 separate donors. CYP activity was measured in quadruplicate for each PHH preparation. % CV (coefficient of variation) is included here as an indicator of assay variability and was calculated using the mean and standard deviation values obtained from the individual donors.

	Luciferin BE (CYP3A)	Luciferin ME (CYP1A2/2C8)	Luciferin ME Su (CYP1A2)	ECOD (CYP1/2/3)
Donors	9	9	8	10
Mean	0.071	1.550	0.816	0.0733
Median	0.051	1.354	0.753	0.047
Range	0.022 – 0.167	0.197-3.044	0.133-1.652	0.018-0.227
% CV	21.2	15.5	13.1	15.2

3.1.1.2.2 Testosterone Metabolism in Freshly Isolated PHH

Testosterone (TST) metabolism was measured in freshly isolated PHH prepared from 3 separate donors and compared by one-way ANOVA. Following 1 hour incubation with substrate, significant donor dependent variations in substrate metabolism ($P<0.001$) and metabolite production ($P<0.05$) were observed (Figure 3-2).

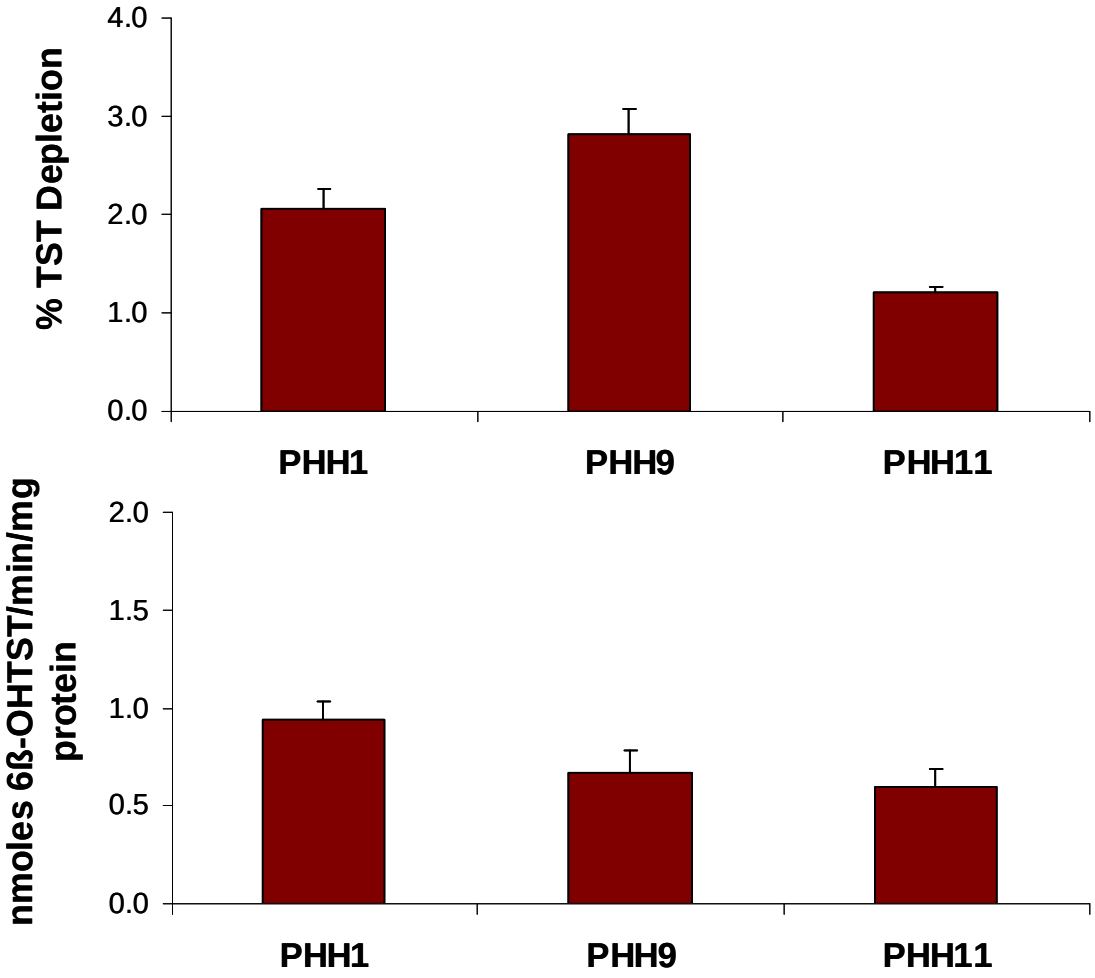


Figure 3-2. (top) % substrate depletion and (bottom) 6β-OHTST production were measured by HPLC analysis of supernatant obtained from freshly isolated PHH from 3 separate donors incubated for 1 hour with 200µM TST.

Between donors, the extent of substrate depletion did not always correspond with extent of 6β-OHTST metabolite production – PHH9 demonstrated the greatest level of TST depletion but a lower level of 6β-OHTST production than PHH1. One likely explanation for this was alternative metabolite production by additional CYP isoforms (other than CYP3A4). An example UV trace, obtained following HPLC analysis of

supernatant from PHH incubated with TST, is shown in Figure 3-3. Testosterone and 6β-OHTST are shown at ~ 16 and 9 minutes respectively. An additional peak is also highlighted which appeared only when PHH were incubated with TST and demonstrated a similar UV spectrum to TST and 6β-OHTST (detected at 247nm).

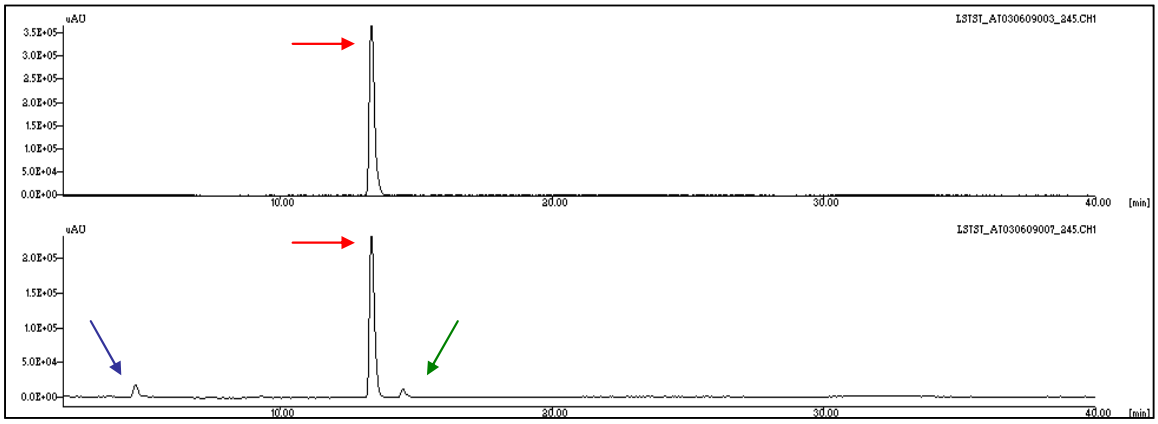


Figure 3-3. HPLC analysis of (top) culture medium spiked with 200µM TST and (bottom) culture medium spiked with 200µM TST and incubated for 1 hour with freshly isolated PHH. Presence of TST (→), and 6β-OHTST (→) were confirmed by analysis of standards in the same run. An additional peak (→) was also observed only when PHH were incubated with TST.

LC-MS was used to analyse this peak. In order to remove any residual salt contamination not eluted within the first minutes of LC analysis, a sample (500µl) of the assay supernatant from PHH was extracted with chloroform and concentrated by resuspending in 100µl of 15% ACN. This sample was then subjected to LC-MS analysis using the same solvent gradient defined for HPLC analysis (Chapter 2) but with a 0.3µM column and a flow rate of 0.3ml/min. Consistent results were obtained when supernatant from 2 separate donors was analysed. The later peak was confirmed as having a molecular weight of 323. TST has an MW of 288, therefore it is not a product of TST breakdown, nor was it an adduct of TST and ACN. Primary β metabolites of testosterone have a MW of 304. Potentially this peak is a product of metabolite conjugation. Since it only appears when cells are incubated in the presence of TST it is likely a product of TST metabolism, however, since LC-MS analysis was inconclusive, fraction collection and GC-MS analysis would be required to confirm this.

3.1.1.2.3 Culture Conditions for Adult Primary Human Hepatocytes

In cultured hepatocytes, a large donor dependent variation in hepatocyte attachment and CYP function was observed. For individual donors this was also influenced by culture conditions.

3.1.1.2.3.1 Attachment and Confluency of Cultures

Following overnight (16h) attachment, confluency of hepatocyte cultures was assessed by phase contrast microscopy and examples are shown in Figure 3-4. A large variation in attachment was observed between donors and exclusion criteria were developed whereby for monolayer function studies, only hepatocyte cultures with greater than 50% attachment, which formed good cell-cell connections (minimum doublets) were included.

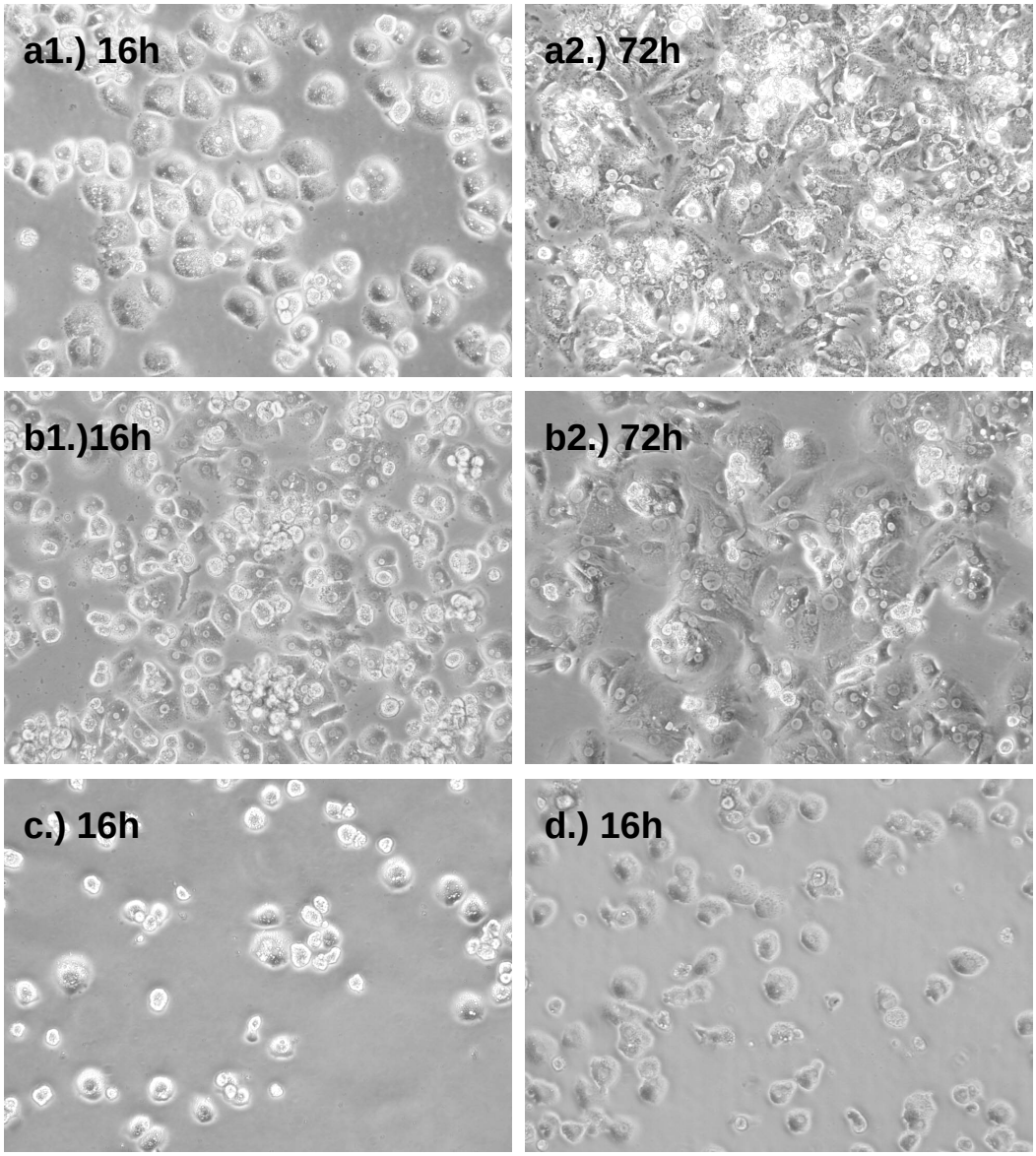


Figure 3-4. Examples of hepatocyte cultures (a1) PHH 10 and (b1) PHH 29 with good overnight attachment and cell-cell connections which (a2) / (b2) could be used for experiments requiring prolonged culture, and hepatocyte cultures (c) PHH 34 and (d) PHH 2 with poor overnight attachment and minimal cell-cell connections which were excluded from monolayer experiments. Time, where indicated, is hours post isolation. Images are x20 magnification.

3.1.1.2.3.2 CYP Activity Declines in Adult Human Hepatocytes During Time in Culture

ECOD activity was measured over up to 8 days post isolation in PHH isolated from 5 separate donors (PHH 32, 30, 31, 10, 3) plated out in AHM with 10% FCS. In each instance CYP activity declined during prolonged culture (Figure 3-5). The greatest drop in activity (47+/-6%) was seen between days 1 and 2 post isolation. Between days 5 - 8 function settled around 10-20% of initial measured activity.

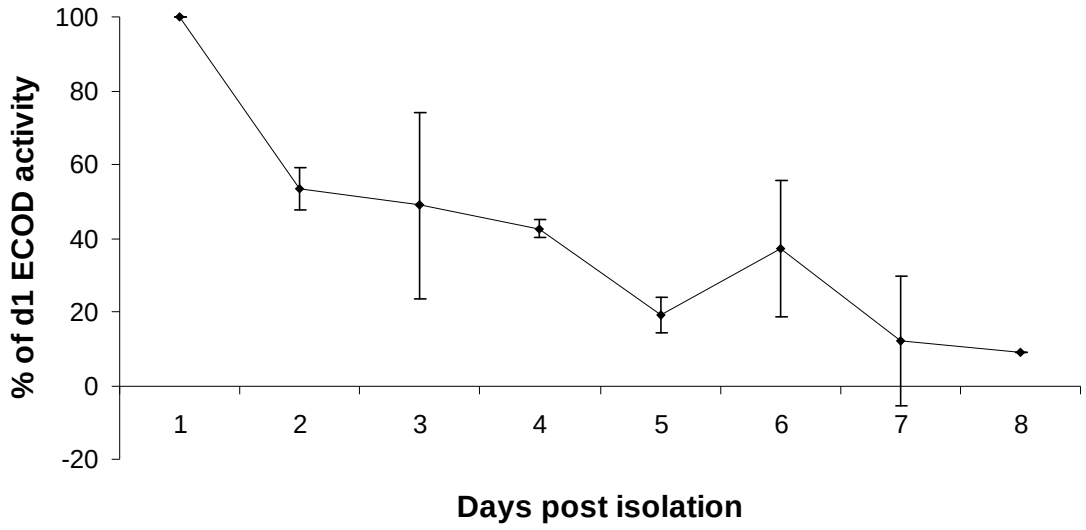


Figure 3-5. Cytochrome P450 (ECOD) activity declines in PHH during prolonged culture. ECOD activity was measured daily over 7 (PHH 31, PHH 30, PHH 10, PHH 3) or 8 (PHH 32) days and final results were calculated as a % of d1 function. Data is presented as an average of donors +/-sd.

3.1.1.2.3.3 Effect of Growth Factors on Cytochrome P450 Activity

PHH were cultured for 48 hours in basal WEM or in WEM + HGF (20ng/ml) and EGF (10ng/ml). CYP activity was measured in PHH from multiple donors by either Luciferin ME, Luciferin BE or ECOD assay and then for each assay type data was analysed by ANOVA.

Luciferin ME metabolism was measured in PHH isolated from 3 separate donors cultured +/- growth factors and data is shown in Figure 3-6 (a). A significant donor dependent variation in CYP1A2 activity ($P < 0.001$, $F(2, 4242) = 936$) was observed in each instance (basal range: 1.36-36.78 pmoles d-luc/min/mg protein), however for each PHH preparation, a significantly lower ($P<0.01$, $F(1, 23.63) = 10.5$) level of CYP1A2 activity was measured in PHH cultured in the presence of growth factors. The extent to which growth factors decreased activity was also found to be donor dependent ($P<0.05$,

F (2, 30.96) = 6.83). Following exposure to growth factors, CYP1A2 activity was decreased by 43% in PHH33 and to a lesser extent in PHH32 (16%) and PHH5 (20%).

Luciferin BE metabolism was measured in PHH isolated from 2 separate donors cultured +/- growth factors (Figure 3-6 (b)). In this assay, a large donor dependent variation in activity was observed (basal activities: 0.39/0.08 pmoles d-luc/min/mg protein). Growth factors were without significant effect on CYP3A activity although their effects varied markedly for each donor measured. Following culture with growth factors, CYP3A activity was decreased by 57% in PHH5 and increased by 15% in PHH33.

ECOD activity was measured in PHH isolated from 2 separate donors cultured +/- growth factors (Figure 3-6 (c)). In this assay, a large donor dependent variation in activity was observed (basal activities: 0.004/0.39 pmoles d-luc/min/mg protein, however growth factors had no significant effect on ECOD activity. Following exposure to growth factors, ECOD activity was decreased by 16% in PHH8 and 7% in PHH30 (compared to basal activity).

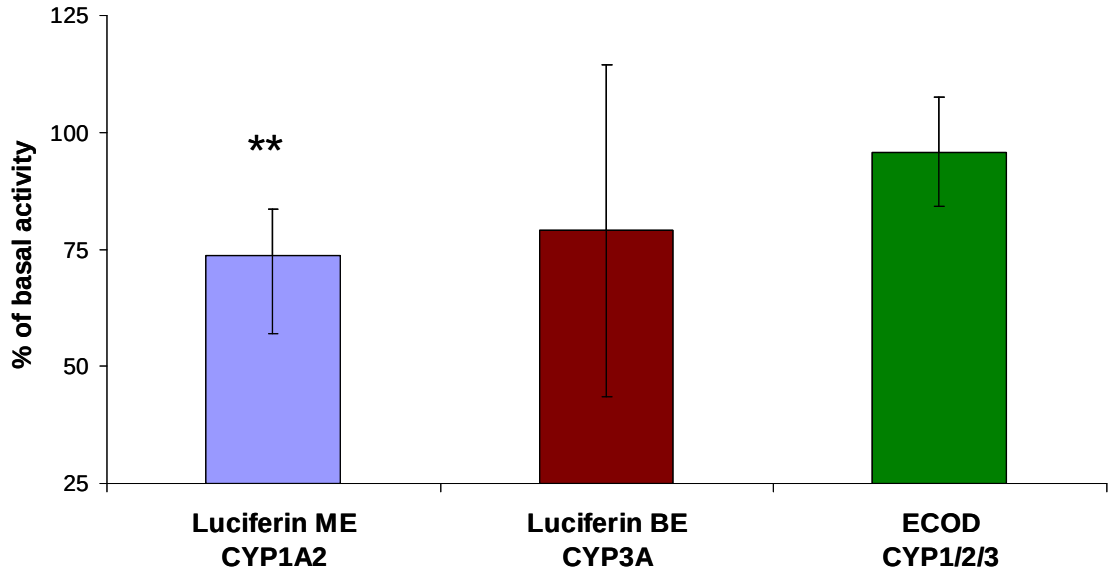


Figure 3-6. CYP function was compared in PHH cultured for 48 hours +/- growth factors. At day 3 post isolation, CYP activity in PHH cultured under basal conditions and in the presence of growth factors (HGF & EGF) was measured in quadruplicate for each donor. The % difference between the two conditions was then calculated and is expressed as mean +/- range of 2 (CYP3A:PHH5/PHH 33 and ECOD: PHH8/PHH30) or 3 (CYP1A2: PHH5/PHH32/PHH33) different donors. Following two-way ANOVA ** P < 0.01 when P450 activity in presence and absence of growth factors was compared.

3.1.1.2.3.4 Effect of Serum or Plasma on Cytochrome P450 Activity

ECOD activity was measured up to 2 days post isolation in PHH isolated from 3 separate donors which had been plated out and cultured in WEM containing either 10% FCS or 10% (human) FFP. Although ECOD activities were significantly different between donors ($P < 0.001$, $F(2, 0.58) = 99.1$), the differences in ECOD activity between PHH cultured in FCS and FFP were negligible for each donor (Figure 3-7 top). However there was a slight difference observed between FCS and FFP when levels of activity measured at d1 and d2 post isolation were compared (Figure 3-7 bottom). Although the effect was donor dependent and was not significant for any of the PHH preparations measured, ECOD activity decreased less between d1 and d2 when PHH were plated and cultured in FFP (35-71% decrease) than in FCS (57%-110% decrease).

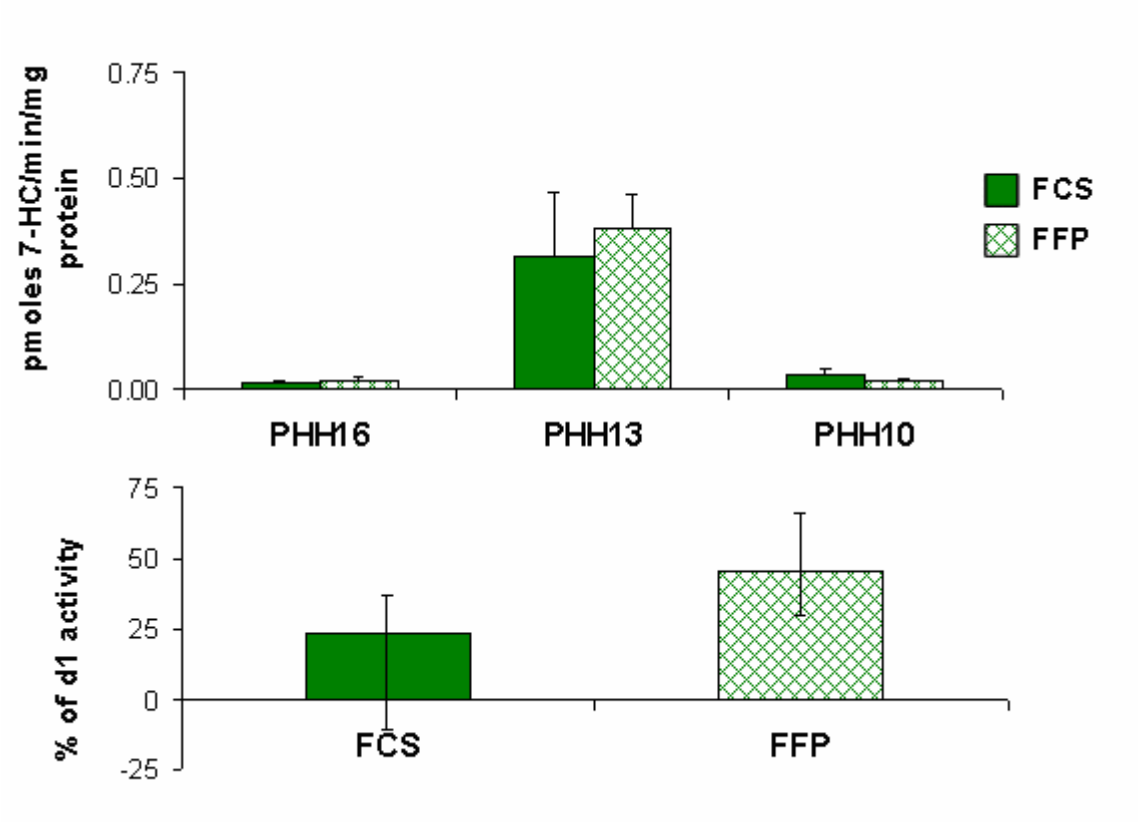


Figure 3-7. ECOD activity was compared in PHH that were plated and then cultured in 10% FCS or 10% FFP. ECOD activity was compared d1 post isolation for the two serum types in PHH isolated from 3 separate donors (top, n=4+/-sd). For the same 3 donors, decline in ECOD activity between day 1 and day 2 was then calculated for PHH cultured in each medium type and is presented as mean of 3 donors +/- range (bottom).

3.1.1.2.4 Cytochrome P450 Activity is Maintained in PHH by Chemical Induction

Effect of inducers on CYP function was considered in the presence of FCS, FCS + heparin control or FFP and measured by ECOD activity. The effect of Phenobarbital on CYP2B6 and CYP3A4 function in PHH was then measured by BOMFC metabolism.

3.1.1.2.4.1 Influence of Serum or Plasma on CYP Induction

The effect of serum on CYP induction was considered in PHH isolated from two separate donors (PHH1, PHH19). Cells were incubated with CYP inducers in WEM containing 10% FCS, 10% FCS with heparin or 10% FFP (O-ve pooled from multiple donors). Following 48 hour induction, CYP function was determined by ECOD activity and fold induction above vehicle determined. For both donors, there were significant differences in P450 induction ($P < 0.05$, $F(2, 0.181) = 4.40$) measured in the presence of FCS +/- Heparin and FFP.

Overall, effects of inducers on ECOD activity were serum and donor dependent (Figure 3-8). There was an effect of heparin on DBA mediated induction for both donors; in the presence of FCS, 10µM DBA resulted in a 1.3 fold increase in ECOD activity (above vehicle) which was inhibited by the addition of heparin or by (heparinised) FFP. Effects of indirubin (20µM) mediated induction were donor dependent; this inducer was without effect on PHH1 cultures. There was slight induction (1.3 fold) measured in PHH19 in the presence of FCS which was diminished in the presence of FCS and heparin or FFP. For both donors there was an effect of FFP on dexamethasone mediated induction; ECOD activity was diminished slightly by 50µM dexamethasone in the presence of FCS +/- heparin (0.83/0.83 fold compared to vehicle activity), but was increased (1.22 fold) in the presence of FFP. For both donors, ECOD activity was not improved by rifampicin (50µM) in the presence of FCS +/- heparin. This was unchanged by FFP for PHH19, however for PHH1 rifampicin induction in the presence of FFP resulted in a 1.46 fold increase in ECOD activity above vehicle treated cells.

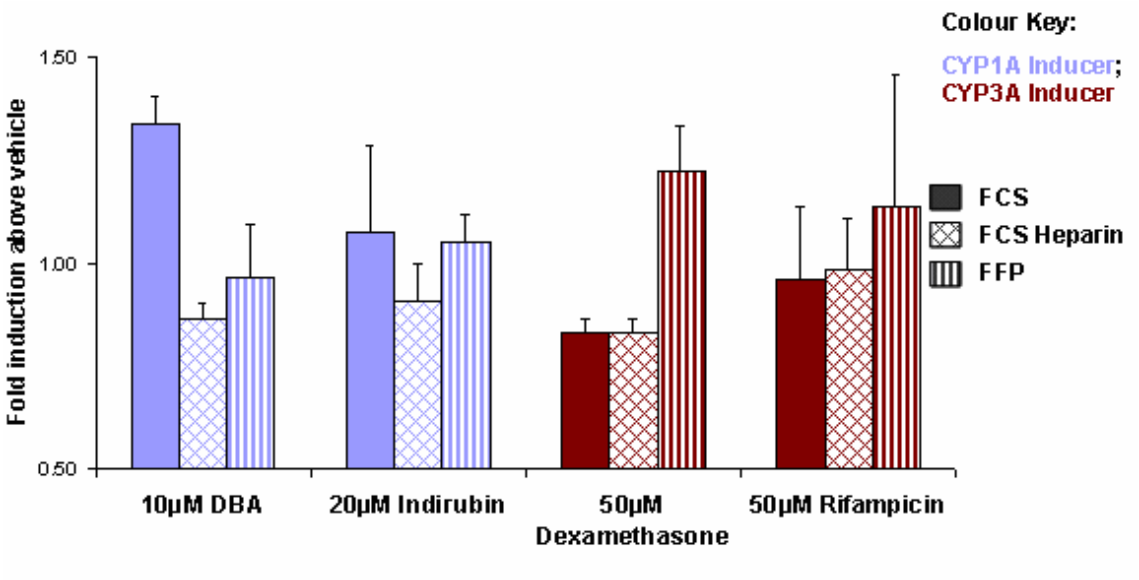


Figure 3-8. ECOD activity was measured in PHH1 and PHH19 following 48 hour induction with CYP inducers in WEM containing 10% FCS, 10% FCS and Heparin or 10% FFP. For each inducer fold induction in CYP activity above vehicle was calculated and is expressed as the average of the two donors +/- range.

3.1.1.2.4.2 Vivid BOMFC Metabolism and Phenobarbital Induction

Vivid® BOMFC metabolism can be used to assess the function of CYP3A4 and CYP2B6 and was measured in monolayer cultures of PHH18 at either d1 post isolation or d3 post isolation following 48 hour incubation with phenobarbital. Substrate metabolism was measured in the presence or absence of 100µM clarithromycin which should inhibit CYP3A4 function at this concentration.

Substrate metabolism was significantly affected by phenobarbital induction ($P < 0.05$, $F(2, 3.76) = 5.61$) and the presence of inhibitor ($P < 0.001$, $F(1, 8.50) = 25.33$).There was also a significant interaction between the two variables ($P < 0.01$ $F(2, 5.06) = 7.51$); phenobarbital induction was diminished in the presence of inhibitor although activity in uninduced controls was comparable between groups.

Bonferroni post tests were used to compare BOMFC metabolism at d1 post isolation and d3 post isolation following induction (Figure 3-9).

When measured in the absence of inhibitor, there was a significant decrease in BOMFC (CYP3A4/CYP2B6) metabolism between d1 and d3 post isolation (1.6 fold $P < 0.01$). This difference was abrogated following phenobarbital induction which resulted in a

significant increase (1.7 fold P<0.01) in PHH function above 0.1% DMSO vehicle treated cells.

In the presence of clarithromycin, BOMFC metabolism (CYP2B6) following 48 hour induction with phenobarbital was slight compared to vehicle treated (uninduced) cells, however, phenobarbital incubation did result in a significant improvement in function (1.9 fold P<0.05) relative to d1 PHH which was greater than the effect of vehicle alone.

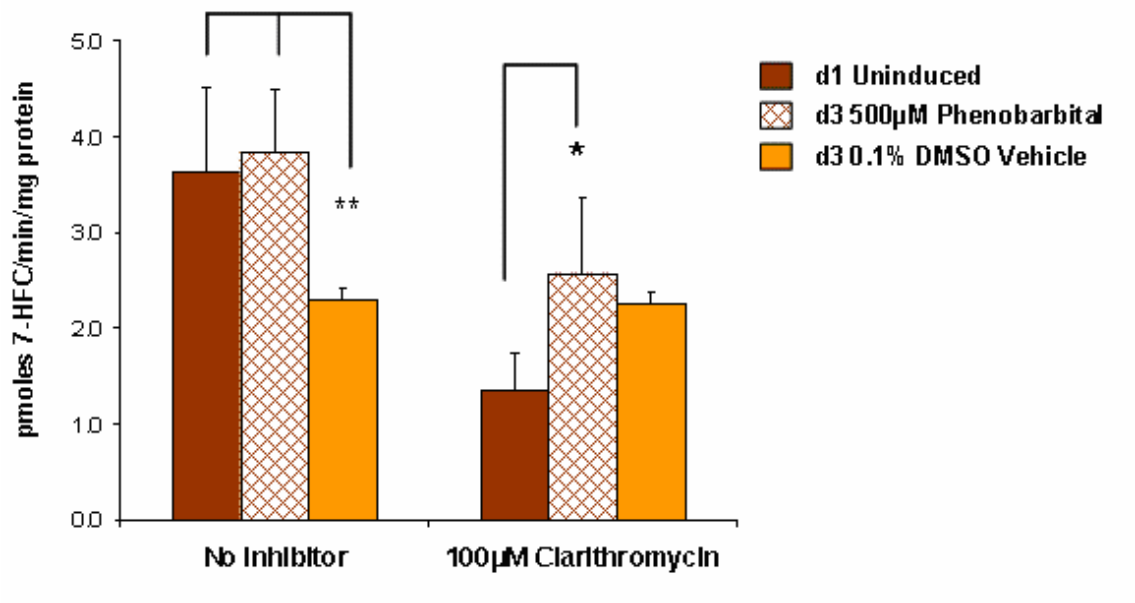


Figure 3-9. BOMFC metabolism was measured +/- 100µM clarithromycin in PHH13 either d1 post isolation or d3 post isolation following 48 hour incubation with 500µM Phenobarbital or 0.1% DMSO vehicle.

Following two-way ANOVA and Bonferroni post test, ** P < 0.01, * P < 0.05 (n=4+/-sd).

3.1.1.3 Discussion

In summary, experiments performed on hepatocytes derived from a number of livers demonstrated a large variation in CYP activity between donors.

CYP function was measured in freshly isolated PHH isolated from up to 10 donors by Luciferin BE (CYP3A), Luciferin ME (CYP1A2/2C), and ECOD assays. The ranges of these values were summarised in Table 3-2 and these will be referred to throughout this thesis. A metabolism profile of testosterone (TST) by freshly isolated PHH was also determined and levels of TST depletion and 6β-OHTST production measured for a number of donors.

In freshly isolated PHH, Luciferin ME derived luciferase activity, assessed both with and without the CYP2C inhibitor sulfaphenazole, was higher than Luciferin BE derived luciferase activity, implying that potentially CYP1A2 function was dominant in all these samples. Although cells used in this study were obtained from normal tissue, thus are not carcinoma derived, they were not obtained from a healthy population and potentially their CYP function could be affected by disease status. A limitation of the hepatocytes used in this thesis is the lack of clinical data on the donors that was made available. In particular, drug therapy (including chemotherapy regimen) and smoking could both contribute to variability in CYP function.

As an additional consideration, as described in Chapter 1, Luciferin ME is not an entirely specific substrate, and is also metabolised by several other CYP isoforms (particularly CYP4A11 and CYP2C8/9) which are involved in endogenous and exogenous metabolism. This will be considered in future chapters when comparing data from these assays.

It is well established that a number of factors including genetics, diet and environmental influences will alter an individual's CYP expression, however, it is also reported that various artefacts associated with isolating hepatocytes and maintaining them in culture can have an effect [63, 65, 222]. Attachment of PHH and therefore confluency varied for each donor which may have affected CYP function. The effect of time in culture and presence of growth factors, serum and inducers on CYP activity in human hepatocytes was demonstrated here. Potentially culture media components such as serum in addition to CYP inducers could be varied within a BAL to achieve both enhanced cell growth and improved hepatic function. In PHH, however, effects of media components and CYP inducers were both found to be donor dependent.

CYP activity was therefore examined in human liver tissue or freshly isolated PHH to assess variation in CYP function independent of hepatocyte preparation and culture.

3.1.2 Human Liver Microsomes

Human liver microsomes (HLM) were isolated from a number of donor samples and their CYP function measured to compare interindividual variation in function with that measured in primary human hepatocytes (PHH).

3.1.2.1 Human Liver Microsome Isolation

Where possible, microsomes were isolated from fresh tissue or hepatocytes. In all other cases samples were immediately snap frozen in liquid nitrogen and stored at -80°C until required. These samples were thawed in an appropriate volume of homogenisation buffer at 4°C immediately prior to microsome preparation.

Homogenisation Buffer (pH 7.5)
0.25 M Sucrose
50mM HEPES
100mM NaCl
Complete mini EDTA-free Protease Inhibitor Cocktail Tablets (Roche 04 693 159 00, 1 tablet/10ml buffer)

Resuspension Buffer (pH 8.5)
0.5 M Triethyl ammonium biocarbonate
0.1% SDS (sodium dodecyl sulphate).

(The original intended use for some of these HLM preparations was proteomic analysis which is the reason this resuspension buffer was chosen. For consistency therefore all the HLM preparations were resuspended in this buffer but at a sufficient protein concentration that it was diluted substantially when HLM were added to CYP functional assays).

Potassium Phosphate Buffer (KPO_4) pH 7.4
50mM K_2HPO_4
50mM KH_2PO_4

Beckman ultra-clear centrifuge tubes (14 x 89mm # 344059)
CAT X-120 electronic homogeniser (Finemech)

Liver was finely minced and this or hepatocytes were homogenised on ice in homogenisation buffer (1ml of buffer per 0.1g of tissue or $1.5x10^7$ cells) and microsomes were then isolated by differential centrifugation at 4°C [223]. Initially, homogenate was centrifuged at 1000g for 10 minutes to sediment cell debris, nuclei and unbroken cells. The supernatant from this spin was transferred to fresh microfuge tubes and centrifuged at 12,000g to pellet the mitochondrial fraction. The supernatant was transferred to centrifuge tubes and centrifuged for a third time in an ultracentrifuge at 180,000g for one hour. The resultant pelleted microsomal fraction was resuspended in an appropriate volume of resuspension buffer (determined by estimated yield) and protein content determined by the BCA method. Diluted microsomes were distributed into 50µl aliquots (~250µg protein) which were stored at -80°C until required.

3.1.2.2 Analysis of Cytochrome P450 Function in HLM

CYP activity was assessed in HLM by ECOD or luciferase assays.

3.1.2.2.1 ECOD Activity

20µg of microsomal protein was combined with 7-EC (at a final concentration of 500µM, 2%MeOH) in 100mM KPO_4/ 0.1mM EDTA in polypropylene microfuge tubes in a total volume of 180µl. Tubes were prewarmed to 37ºC for 10 minutes and then 20µl of 10mM NADPH added to initiate the enzymatic reaction. Tubes were gently mixed and incubated in a water bath for 30 minutes. After this time period, tubes were placed on ice and 25µl of chilled 2M HCl was added to terminate the reaction.

Product was then extracted to remove background fluorescence caused by NADPH at similar wavelengths. This was done by adding 900µl of chloroform, mixing well and vortexing .Tubes were centrifuged at 3000g for 1 minute and the product back extracted by transferring 500µl of the lower organic phase to a 1.5ml microfuge tube containing 500µl of 0.01M NaOH/1M NaCl. This mixture was vortexed and centrifuged at 3000g for 1 minute. 200µl of the upper aqueous phase was transferred to a 96 well plate and analysis carried out as described in Chapter 2.

3.1.2.2.2 Luciferase Assays

Quantification of CYP function was carried out using Promega P450 Glo assay system as per manufacturer's directions. Briefly, in white opaque 96 well plates, 20µg of

microsomal protein was combined with 50µM of either Luciferin-ME or Luciferin-BE substrate in 100mM KPO_4 buffer in a volume of 25µl. The plate was sealed and preincubated at 37°C for 10 minutes. 25µl of 200µM NADPH in KPO_4 was added to initiate the reaction and the plate sealed and incubated for 30 minutes at 37°C. The plate was then developed following the method described in Chapter 2.

3.1.2.3 Results

3.1.2.3.1 Comparison Between HLM Isolated From Tissue or Hepatocytes

CYP activity was compared in HLM from a single donor (PHH28) that had been prepared from either freshly resected tissue or freshly isolated PHH (Figure 3-10).

For each assay measured, there were no significant differences observed between activities measured in HLM prepared from tissue or from hepatocytes although there was a lower (1.8 fold) CYP1A2 (Luciferin ME) activity when microsomes were prepared from PHH compared to tissue.

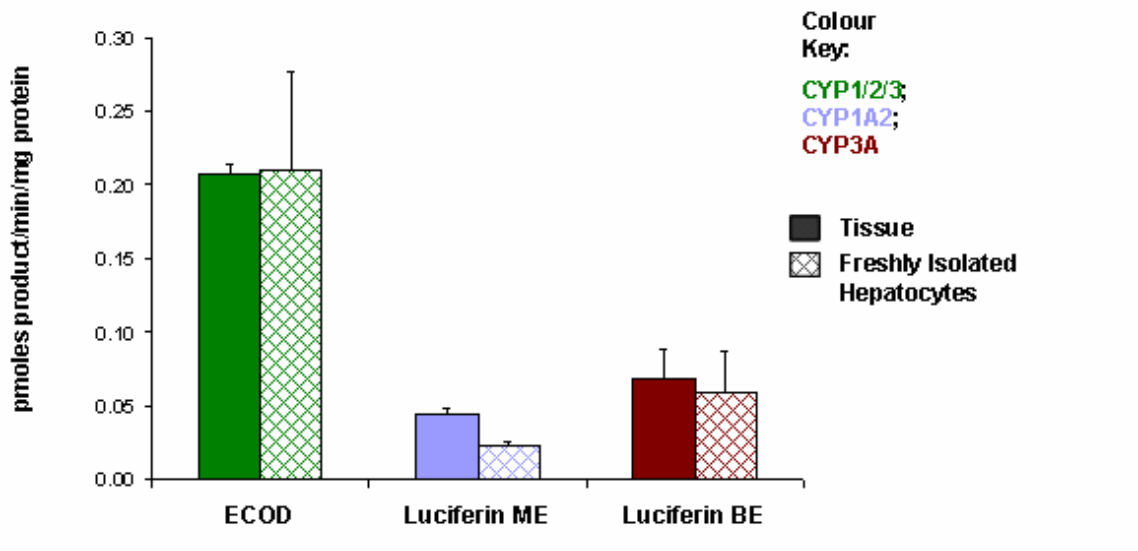


Figure 3-10. CYP activities were compared for HLM from a single donor (PHH28) prepared from tissue (solid bars) or freshly isolated hepatocytes (hatched cells). Individual assays were compared by Student's t test and differences were not found to be significant.

3.1.2.3.2 Comparing CYP Activity Between HLM and Freshly Isolated PHH From the Same Donor

CYP activity was measured in HLM and freshly isolated PHH isolated from the same donor. For each assay type, the interindividual differences in activity between donors were maintained between HLM and PHH (Figure 3-11).

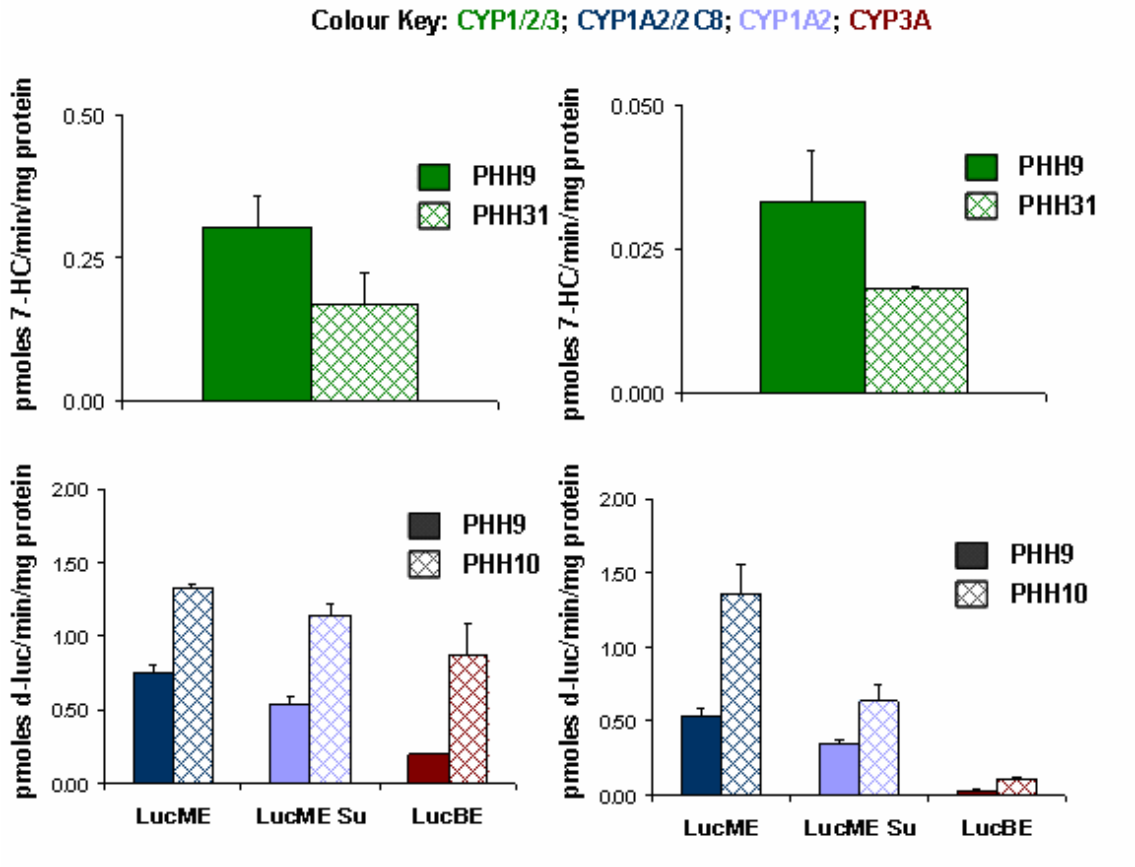


Figure 3-11. Interindividual variation in CYP activity was maintained in (top) ECOD and (bottom) luciferase assays when activities were compared between (left) HLM and (right) freshly isolated PHH isolated from the same donor (n=4+/-sd). Note that Su= measured in the presence of 10µM sulfaphenazole.

3.1.2.3.3 Variations in CYP Activity were Maintained Between HLM and Cultured PHH

CYP activity was measured in HLM and hepatocytes isolated from the same donor and then maintained in culture for 72 hours, during which time CYP activity decreased as observed in section 3.1.1.2.3.

As was observed for comparisons between HLM and freshly isolated PHH, for each assay type, the interindividual differences in activity between donors were maintained between HLM and PHH, although the differences between donors were less pronounced in cultured hepatocytes (Figure 3-12).

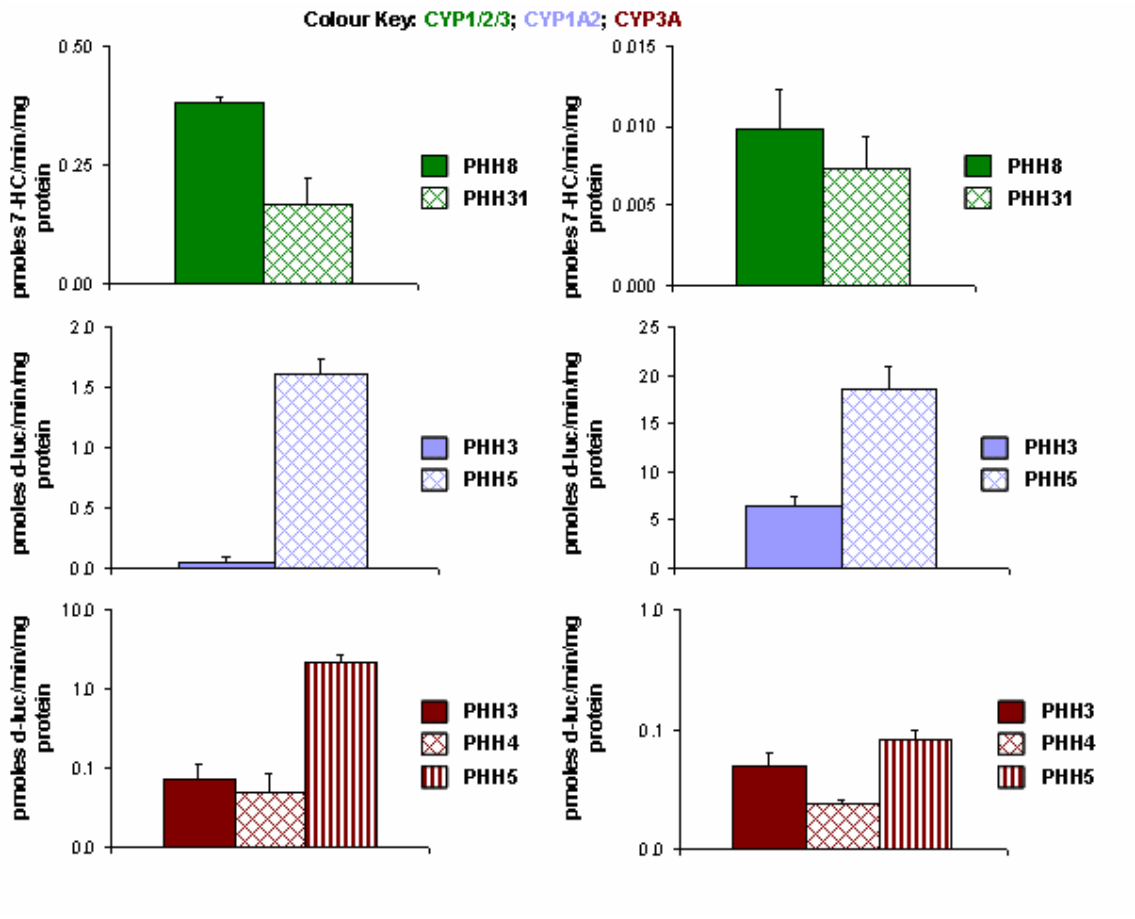


Figure 3-12. Interindividual variation in CYP activity was maintained in (top) ECOD, (middle) Luciferin ME and (bottom) Luciferin BE assays when donor activities were compared between (left) HLM and (right) PHH following 72 hours culture (n=3+/-sd).

3.1.2.4 Discussion

CYP activity was measured in HLM prepared from either liver tissue or freshly isolated PHH and overall CYP function was comparable between the two, although CYP1A2 (Luciferin ME) activity was slightly decreased in HLM from PHH. This data implies that the hepatocyte isolation process does not have a significant effect on CYP activity; this could be further confirmed by expanding this comparison to incorporate multiple donors, but these initial findings validate the use of freshly isolated PHH as an appropriate indicator of CYP function in adult liver to which BAL relevant cell lines can be compared.

CYP activity was then compared using hepatocytes and HLM isolated from the same donor, and this confirmed that the significant variation in CYP activity in hepatocytes isolated from a range of donors was directly related to the individual rather than an artefact of hepatocyte isolation or culture conditions. The differences were more apparent in HLM prepared from tissue and freshly isolated PHH than in hepatocytes that had been cultured for a number of days, most likely due to loss of CYP activity during culture, however, the rank order of levels of CYP activity in both freshly isolated and cultured hepatocytes and HLM assays from individual donors was equivalent for each detection method considered. A similar comparison has previously been made in humans between CYP activity detected *in vivo* and CYP mediated metabolism by primary hepatocytes [224]. The data presented in this report confirms that comparisons made between CYP activity in cultured hepatocytes and HLM are equally valid. This is in agreement with another published study which compared, in rats, CYP inhibition in hepatocytes and microsomes isolated from the same donor [225].

In terms of level of function, HLM assays are not representative of a complete cellular system. Concentrations of enzymes and cofactors used are non-physiological and the amount of available substrate at the enzyme active site differs in microsomal assay systems to hepatocytes. HLM were used here to measure interindividual variation and in this respect, provide invaluable data when considering the range of CYP function between individuals.

3.2 Cytochrome P450 Activity in Human Foetal Liver

Foetal hepatocytes have been considered by other groups for use within a BAL [122, 171] and have been compared to HepG2 cells with regard to their biotransformation properties [20] and so for these reasons CYP function in FHH was considered in this thesis.

3.2.1 Methods

Foetal liver was obtained from the UCL Institute of Child Health. Hepatocytes and non-parenchymal cells were isolated from this tissue using a collagenase digestion protocol modified from Lázaro *et al.*[226].

3.2.1.1 Isolation of Human Foetal Hepatocytes

1x PBS

Digestion buffer (HBSS containing calcium and magnesium supplemented with 20mM HEPES, 0.5% BSA, 50µg/ml ascorbic acid, 4µg/ml insulin, 3mg/ml Collagenase Type IV)

Williams' Medium E (WEM)

Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)

Tissue was washed in PBS before being minced in collagenase digestion buffer. This tissue suspension was digested by gentle agitation at 37°C for 30 minutes. Cells were collected by centrifugation at 32g (400rpm) for 6 minutes at 4°C. The resultant pellet was resuspended in WEM containing 0.5mM EGTA and successively passed through 23- and then 25-gauge needles to obtain a single cell suspension. Cells were washed, resuspended in WEM and counted. Viability was determined by trypan blue exclusion and ranged from 78-86%.

3.2.1.2 Culture of Human Foetal Hepatocytes

Post isolation, cells were resuspended in Foetal Hepatocyte Medium (FHM, Chapter 2) plus 5% FCS and seeded on 48-well collagen coated tissue culture plates ($2.5x10^5$ cells/well in a volume of 300µl). Following an overnight incubation, cells were washed in WEM and medium changed to remove serum. At this point 20ng/ml of both HGF and EGF were added to cultures. Cultures were maintained in a 37°C, 5% CO2 incubator

and medium replenished every other day. Growth factors were removed from medium two days prior to the introduction of inducers.

3.2.2 Results

Luciferin BE metabolism was measured in FHH and data is shown in Figure 3-13. A significant donor dependent variation ($P < 0.05$) was observed in CYP3A activity in monolayer cultures of FHH isolated from 3 separate donors and cultured under basal conditions; all 3 preparations were within the range measured for freshly isolated PHH described in Table 3-2.

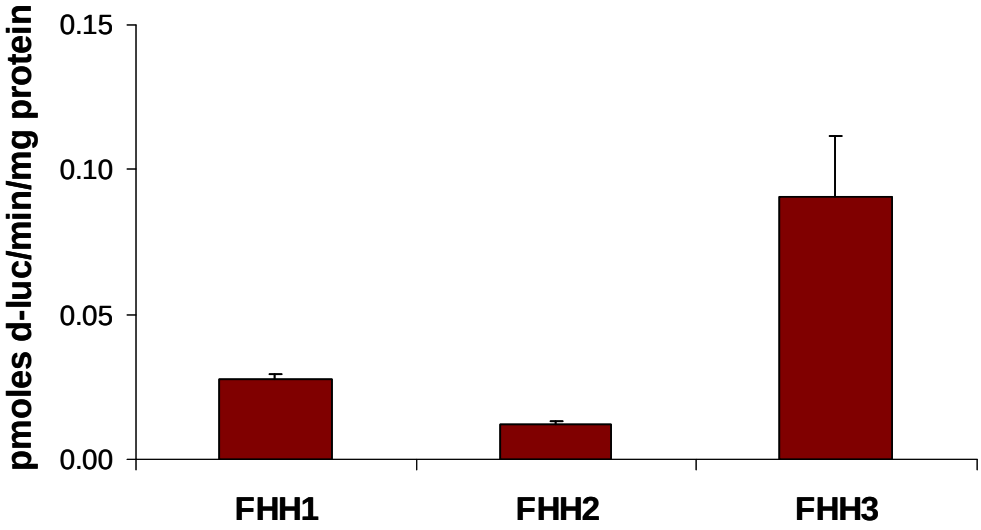


Figure 3-13. CYP3A (Luciferin BE) activity was measured in FHH isolated from 3 separate donors and expanded in culture until confluent (~14 days). For each donor n=3 +/- sd.

Induction of CYP activity was explored in FHH prepared from a single donor (FHH3). Cells were cultured for 96 hours in presence of inducer and CYP3A function was measured by Luciferin BE and testosterone metabolism (Figure 3-14). Although changes in function were not statistically significant, common trends were indicated between the two assays. Following treatment with inducers, Luciferin BE metabolism was induced by dexamethasone (5.2 fold) and to a lesser extent by rifampicin (1.4 fold). Extent of testosterone depletion was also greater in cells treated with dexamethasone (2.2 fold above vehicle) than with rifampicin (0.82 fold). FHH produced detectable quantities of the 6β-OHTST metabolite, although dexamethasone induced cells produced a greater amount than rifampicin treated cells; for both inducers, levels were comparable with vehicle treated cells.

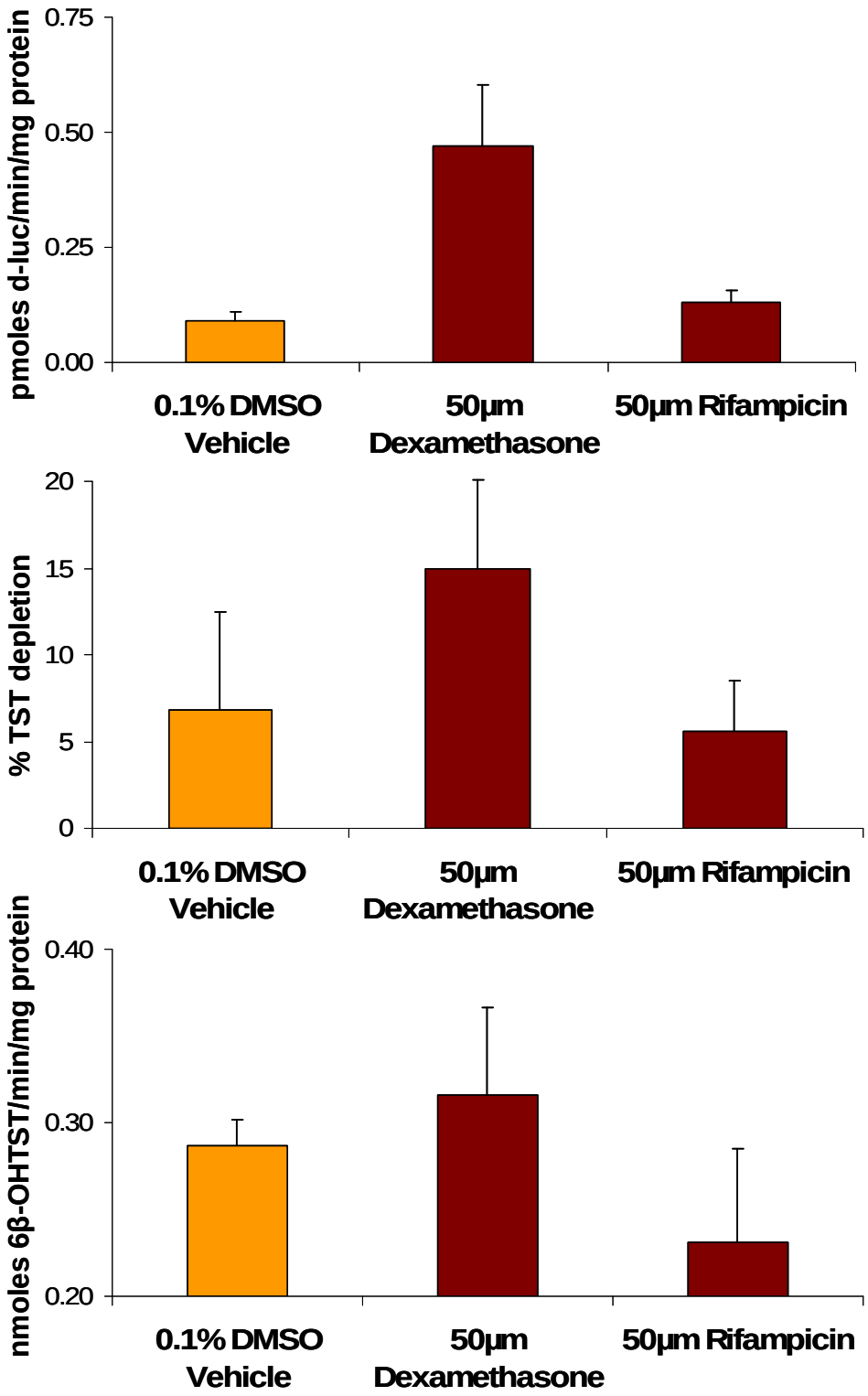


Figure 3-14. FHH (isolated from a single donor-FHH3) were expanded in culture for 10 days post isolation and then treated for 96 hours with 50µM inducers or 0.1% DMSO vehicle. CYP activity was then measured by (top) Luciferin BE assay, (middle) testosterone metabolism and (bottom) 6β-OHTST production. For each assay n=2 +/- range.

3.2.3 Discussion

CYP3A activity was profiled in FHH isolated from 3 separate donors and, like adult PHH, a significant donor dependent variation in function was observed under basal conditions. However FHH differ from adult PHH with regard to their CYP induction. In agreement with other publications, data presented here demonstrated that CYP3A activity is better upregulated by dexamethasone than rifampicin in foetal hepatocytes [138, 164]. In their study, Matsunaga *et al.* found a negligible expression of PXR in FHH and so also considered effects of dexamethasone on CYP3A induction in foetal hepatocytes in the presence of the glucocorticoid receptor (GR) antagonist RU486; it was found that this compound inhibited CYP induction which suggests that CYP3A induction in foetal hepatocytes is GR mediated.

It was demonstrated above that in some circumstances, CYP3A (Luciferin BE) function in FHH matches that measured in some adult PHH. Although the specific isoform mediating this activity was not determined (CYP3A4/5/7) others have demonstrated expression and induction of both CYP3A4 and CYP3A7 in FHH [138]. Further to this, it was shown here that FHH metabolise TST and produce quantifiable amounts of 6β-OHTST which is predominantly produced by CYP3A4. FHH are expandable and express relevant CYPs and so may be a considered as a source of Cytochrome P450 function within a BAL. However, FHH differ from adult hepatocytes not only in their preferential expression of CYP3A7 to CYP3A4 but also in their negligible expression of pregnane X receptor (PXR) [138, 164], which is a key mediator of CYP3A induction. Also, others have demonstrated that CYP functionality in passaged FHH is lower than in isolated adult primary human hepatocytes and although higher CYP activity can be achieved by clonal selection, extensive *in vitro* expansion of FHH may result in decreased hepatic functionality [227]. Consequently obtaining an adequate biomass comprised of foetal hepatocytes could be problematic.

3.3 Conclusions

Here a significant variation in Cytochrome P450 function in human liver was determined for a number of donors and it was established that interindividual changes in CYP function were consistent between PHH and HLM. This emphasises the need to select a number of donors when measuring CYP activity to compare with data obtained from immortalised cell lines.

Other published studies of CYP activity in PHH have used pools of cryopreserved cells from multiple donors (10 is recommended by one cohort [114]) to give a fair representation of CYP function in human liver. Effects of cryopreservation notwithstanding, here CYP activity in PHH from 8-10 individual donors was measured in order that the range of function could be determined rather than an average value alone.

Determinants of CYP function were examined in adult PHH and FHH and differences in CYP induction between cells derived from adult and foetal cells were described. It was also determined that BAL relevant culture conditions including media composition and used of CYP inducers influenced CYP induction, however effects were also donor dependent. These conditions will therefore need to be defined for HepG2 cells and this will be reported in Chapter 4.

CHAPTER 4 Working with What's Available: Adapting HepG2 Culture Conditions to Improve Cytochrome P450 Function within a BAL

Human liver-derived cell lines are widely used as surrogates for primary human hepatocytes as they confer many hepatocyte-specific characteristics. The hepatoblastoma-derived HepG2 cell line is currently being investigated as the potential cellular component of the Liver Group (LG) BAL, and, as discussed in Chapter 1, one system comprising C3A cells, a subclone of HepG2 cells, has been trialed clinically within another BAL device, albeit with limited success. HepG2 cells exhibit many hepatocyte-like functions which may be upregulated by 3D culture, but have lost some of their synthetic and detoxificatory capacity and have thus far been unable to provide the level of CYP function that is required of a BAL.

As described in Chapter 3, CYP enzyme function is variable in primary cells and this is (partly) dependent upon their culture conditions. Moreover, it has been established by others that if culture medium does not support hepatocyte differentiation, a rapid drop of all hepatic-specific functions, including drug-metabolising capacity, occurs. This drop of drug metabolising capacity is (probably) due to the general shutdown of CYP gene expression [115] and this subject has been addressed by several research groups with respect to HepG2 cells [51, 137, 228].

Adjustment of culture format can have positive effects on HepG2 function. In particular 3D culture, resulting in spheroid formation, allows cells to mimic the natural configuration of liver cells *in vivo;* this improves many hepatocyte specific functions [109, 134, 229, 230]. Practically, within a BAL, 3D culture allows a sufficient biomass to be generated in a feasible volume for use in an extracorporeal circuit, which would not be possible in conventional monolayer culture.

LG complete culture medium has been optimised for HepG2 BAL culture to promote cell growth and hepatocellular differentiation; potentially this could have an effect on CYP induction in HepG2 cells. We have also demonstrated that culture medium containing 10% human fresh frozen plasma (FFP) in place of FCS results in enhanced cell proliferation and improved per bead performance in 3D culture. From a regulatory

perspective, use of FFP rather than FCS in culture medium is advantageous since use of animal derived products within a BAL would be undesirable. Furthermore, within a BAL, HepG2 cells are exposed to liver failure plasma (LFP) for periods of up to 8 hours. Others have demonstrated effects of LFP on CYP function as discussed in Chapter 1. Exposure to FFP during HepG2 culture could influence CYP function and this will be explored here.

The aim of Chapter 4, therefore, is to measure CYP induction in HepG2 cells in LG BAL culture conditions in monolayer cells and in encapsulated Hep G2 cells, cultured in static culture, or within a microgravity environment. The effect of exposure to human plasma on CYP activity in HepG2 cells was also considered in both monolayer and 3D culture systems.

4.1 Chemical Induction of Cytochrome P450 in Monolayer HepG2 cells

4.1.1 Methods

HepG2 cells were seeded in 96 well plates at a density of $2.5x10^4$ cells/well in a volume of 100µl complete culture medium (Chapter 2). Following overnight attachment, cells were treated with CYP inducers for 48 hours and then CYP activity was measured by ECOD activity, Luciferin BE metabolism or Luciferin ME metabolism (+ 10µM sulfaphenazole). Cell viability following exposure to inducers was measured by WST-1 assay. Cell protein was determined by the BCA assay.

4.1.2 Results

Each monolayer culture experiment was repeated three times in triplicate and the data presented here are the mean values of the three experiments.

4.1.2.1 Effect of Inducers on ECOD Activity

ECOD activity was measured in HepG2 cells following exposure to inducers over a range of concentrations (Figure 4-1). Following treatment with DBA and indirubin, ECOD activity in HepG2 cells was comparable with lower levels measured in freshly isolated PHH.

Significant induction was achieved in HepG2 cells following treatment with DBA ($P<0.05$), indirubin ($P < 0.001$) and rifampicin ($P < 0.001$). Effects of inducer were

concentration dependent; highest responses were observed with 10µM and 5µM of DBA, 20µM and 40µM indirubin and 50µM and 100µM rifampicin. Dexamethasone treatment did not cause induction of ECOD activity.

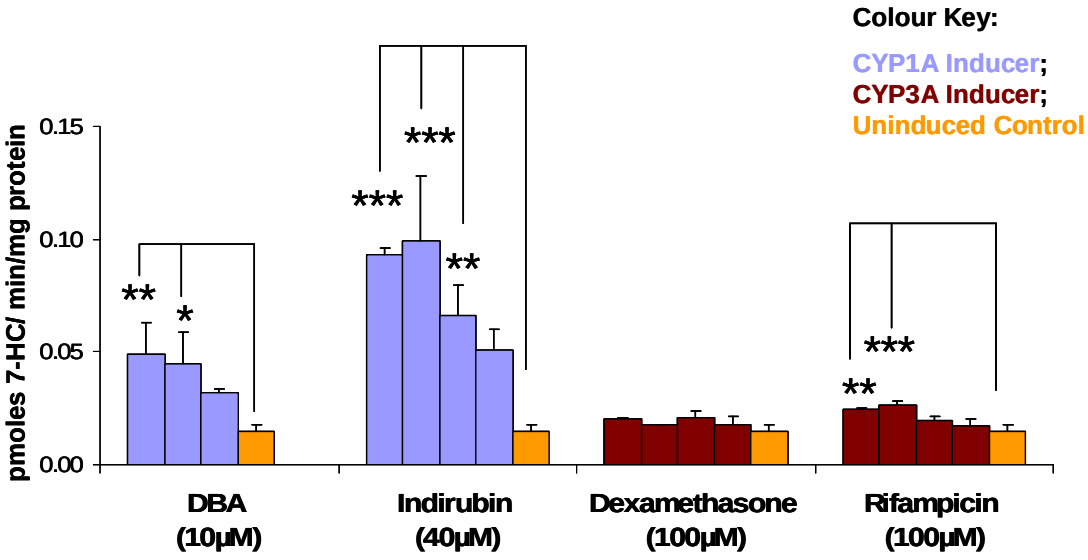


Figure 4-1. HepG2 cells were treated for 48 hours with decreasing concentrations of CYP inducers (1:2 dilution from top concentration indicated in brackets) and CYP function then measured by ECOD assay. Data is expressed as mean (n=3) +/- sd and was analysed by one-way ANOVA followed by Bonferroni post tests. *** P < 0.001, ** P < 0.01, * P < 0.05 when compared to 0.1% DMSO vehicle control (represented by orange bars).

4.1.2.2 Effect of Inducers on CYP1A2 (Luciferin ME) Activity

Luciferin ME metabolism was measured in the presence of 10µM sulfaphenazole and data is shown in Figure 4-2. Treatment with CYP1A inducers resulted in improved function.

There was a significant increase in CYP1A2 activity following 10µM DBA treatment (70.8 fold above vehicle, P < 0.001); activity was also increased to a lesser extent following indirubin exposure (8.1 fold). CYP3A inducers dexamethasone, rifampicin and phenobarbital were without effect on CYP1A2 activity following 48 hour treatment.

Even following DBA induction, function was markedly lower than measured in PHH for this assay.

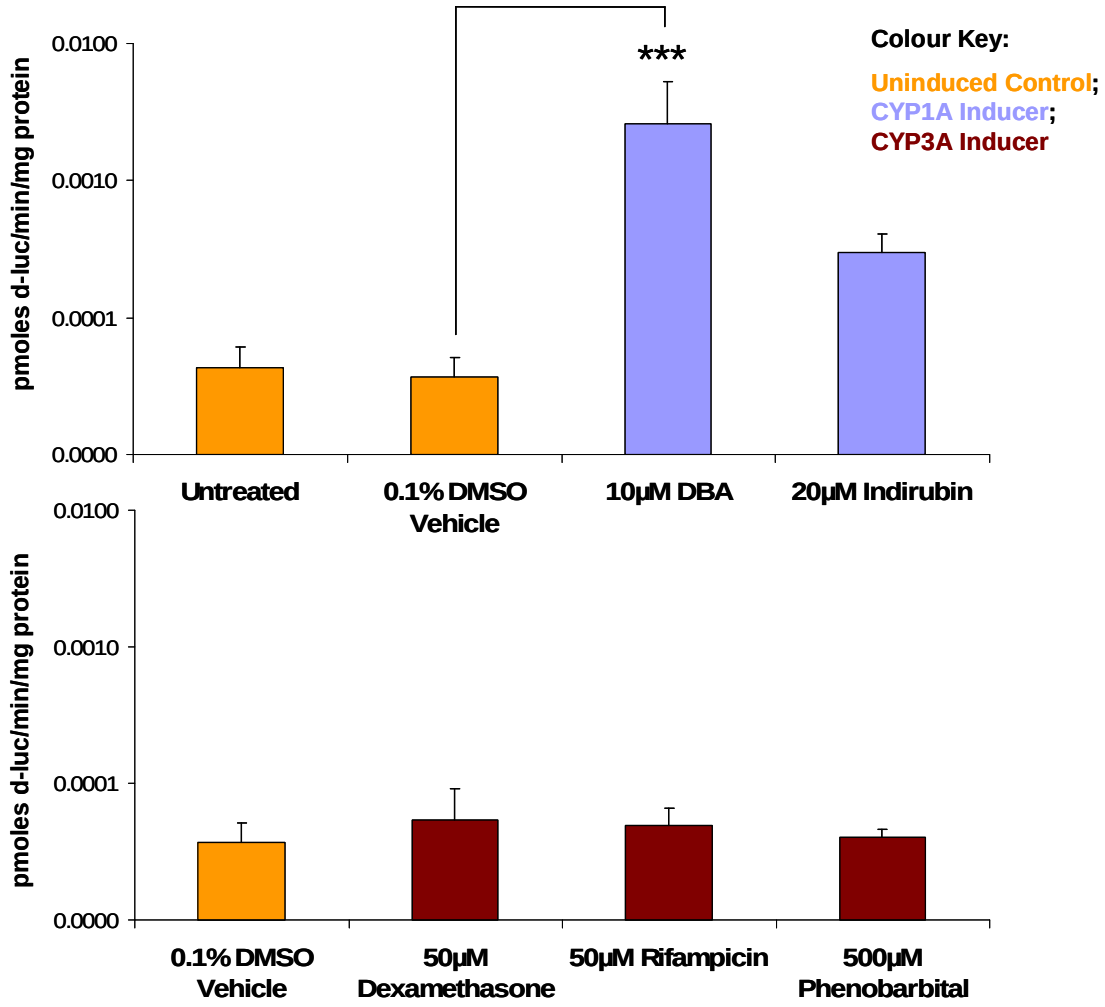


Figure 4-2. CYP1A2 (Luciferin ME) activity was measured in the presence of 10µM sulfaphenazole in HepG2 cells following 48 hour treatment with (top) CYP1A and (bottom) CYP3A inducers. Data is expressed as mean (n=3) +/- sd and was analysed by one- way ANOVA and Bonferroni post test.

***** P < 0.01 when compared to 0.1% DMSO vehicle control.**

4.1.2.3 Effect of Inducers on CYP3A (Luciferin BE) Activity

Effects of inducers on CYP3A (Luciferin BE) activity are shown in Figure 4-3. There was a differential effect of CYP3A inducers on Luciferin BE metabolism. Dexamethasone and phenobarbital did not induce CYP3A function in HepG2 cells by a significant amount, whereas treatment with rifampicin resulted in a significant increase in CYP3A function ($P < 0.01$, 1.7 fold above vehicle). This activity was still lower (15%) than the lowest levels measured in PHH. CYP3A activity was also improved following treatment with indirubin ($P< 0.05$, 1.5 fold).

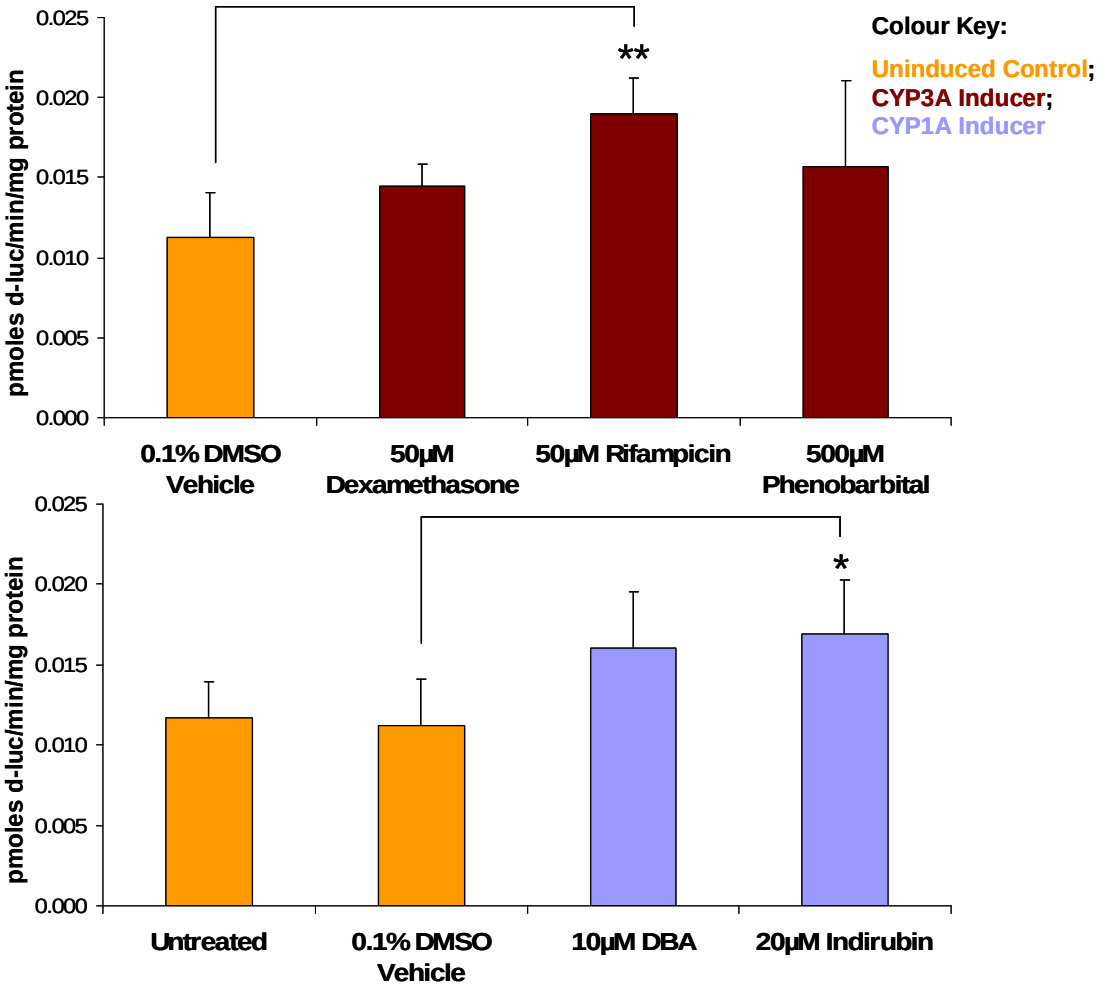


Figure 4-3. CYP3A (Luciferin BE) activity was measured in HepG2 cells following 48 hour treatment with (top) CYP3A and (bottom) CYP1A inducers. Data is expressed as mean (n=3) +/- sd and was analysed by one- way ANOVA and Bonferroni post test. * P < 0.05, ** P < 0.01 when compared to 0.1% DMSO vehicle control.

4.1.2.3.1 Luciferase Assay Selectivity

CYP3A induction was compared in HepG2 cells following measurement with either Luciferin BE or Luciferin PFBE substrate. Experimental conditions were the same for each substrate and were described in Chapter 2. As shown in Figure 4-4, fold induction above basal activity was comparable for each inducer for the two substrates.

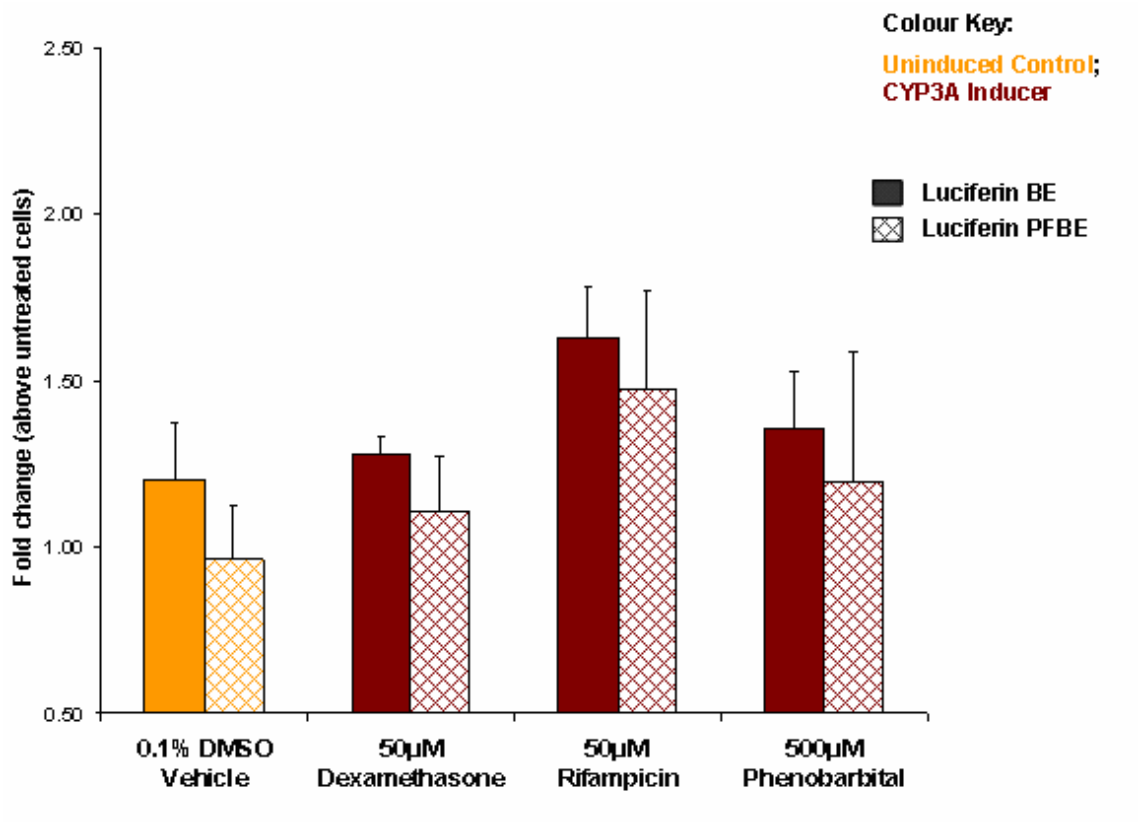


Figure 4-4. CYP3A induction in HepG2 cells was measured using Luciferin BE or Luciferin PFBE substrate following 48 hour exposure to a range of inducers. Fold induction above untreated cells was measured for each inducer and values (n=3+/- sd) are presented for each substrate.

4.1.2.4 Effect of Inducers on WST-1 Activity

WST-1 activity was measured in HepG2 cells to determine the effect of 48 hour induction on cell viability and it was determined that, in HepG2 cells, exposure to any of the inducers measured did not affect WST-1 activity (Figure 4-5).

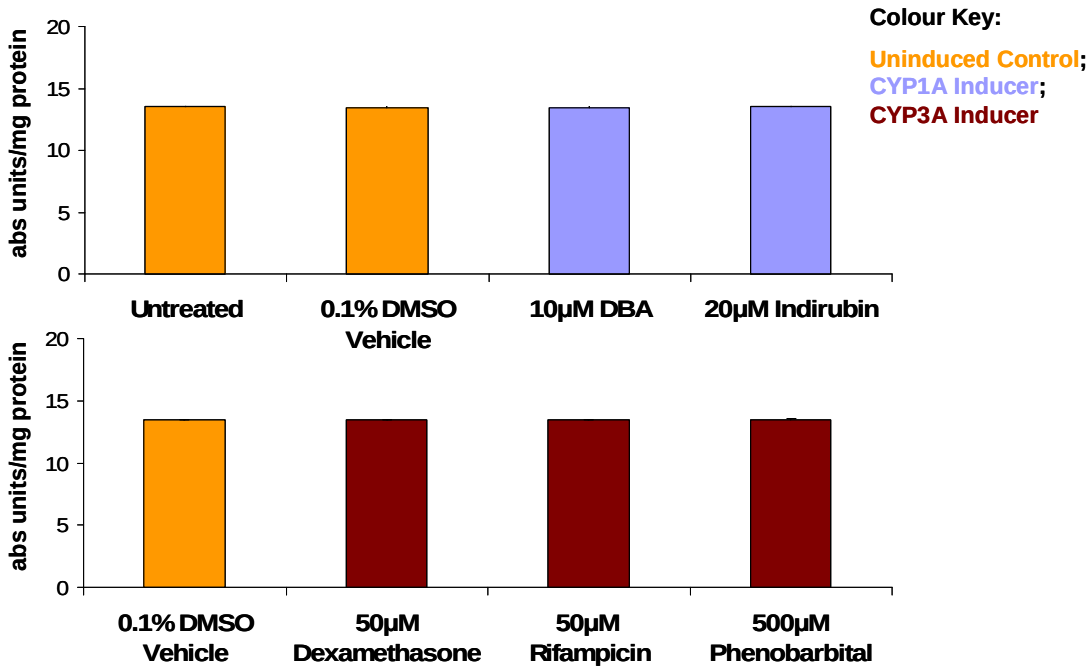


Figure 4-5. WST-1 activity was measured in monolayer HepG2 cells following 48 hour exposure to inducers (n=3 +/-sd).

4.1.2.5 Effect of DBA on Cell Proliferation

Although overall WST-1 activity was not affected by exposure to any inducer, it was evident by microscopy that DBA exposure had an effect on cell proliferation. This was roughly quantified by comparing total protein content in induced and vehicle treated cells.

HepG2 cells were treated for 48 hours with either 0.1% DMSO vehicle or 10µM DBA. Cells were then washed twice with HBSS and culture medium without inducer added. Total protein content was determined by BCA assay 0-72 hours following removal of inducer.

Following two-way ANOVA, there was a significant interaction between time in culture and DBA exposure on cell proliferation ($P < 0.001$, $F(3, 1281) = 45.76$). Exposure of HepG2 cells to DBA resulted in inhibition of cell proliferation and this was not improved even 72 hours after inducer was removed from culture medium (Figure 4-6).

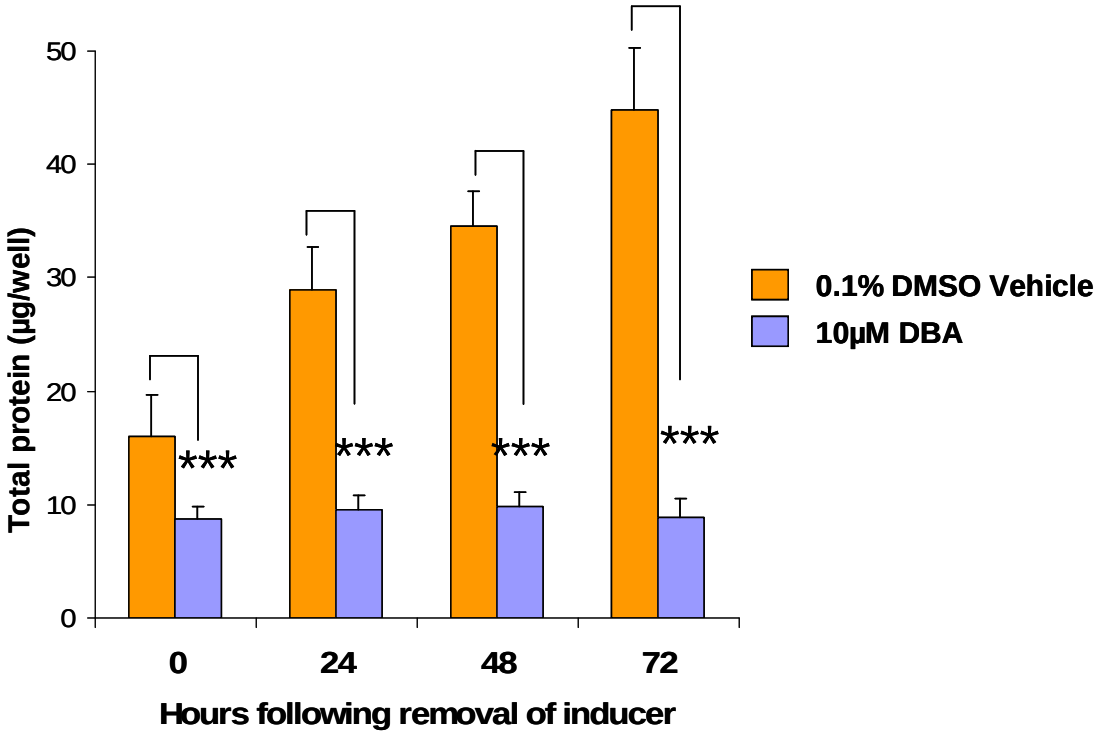


Figure 4-6. Total cell protein (measured by BCA assay) was compared up to 72 hours post induction for HepG2 cells treated for 48 hours with 10µM DBA or 0.1% DMSO vehicle. Following two-way ANOVA and Bonferroni post-test *** $P < 0.001$ when vehicle treated and induced cells are compared (n=3 +/- sd).

4.1.3 Discussion

The initial aim of Chapter 4 was to determine suitable concentrations of CYP inducers required to treat HepG2 cells in order to obtain induction of CYP function over a 48 hour time period. This was firstly measured by ECOD activity over a range of concentrations and it was found that by treating HepG2 cells with indirubin (20µM) or DBA (10µM), activity approached lower levels measured in freshly isolated PHH. Based on ECOD response, a suitable concentration of each inducer was selected to measure induction by luciferase assays.

CYP3A (Luciferin BE) activity was lower in HepG2 cells than PHH although there was a positive effect of induction. Following rifampicin (50µM) induction, HepG2 cell CYP3A activity was improved above uninduced cells although this level was 15% lower than the lowest activity measured in PHH. Of the CYP3A inducers considered, rifampicin treatment resulted in the greatest improvement in CYP function. Others have also indicated that in humans rifampicin is a far greater inducer of CYP3A4 than dexamethasone or phenobarbital [231].

The greatest increase in CYP1A2 (Luciferin ME) activity was measured following DBA treatment (10µM). However even after this induction, the level of function achieved was substantially less (50%) than the lowest level measured in freshly isolated PHH.

In addition to CYP1A2 activity, Luciferin ME assay also measures CYP2C function and so was therefore measured in the presence of 10µM sulfaphenazole, which has been shown, in Chapter 3 and by others [136], to diminish CYP2C mediated Luciferin-ME metabolism. As discussed in Chapter 1, initially the Luciferin ME assay was marketed as a CYP1A2/2C8 specific assay. It was subsequently determined that this assay also detects other CYP isoforms most notably CYP4A11 which is expressed in the liver and is responsible for fatty acid and arachidonic acid metabolism. Potentially this isoforms could be active in PHH and not HepG2 cell which could account for the discrepancy in function between the two cell types.

Different effects of the CYP1A inducers indirubin and DBA were also shown here between ECOD and Luciferin ME assays. DBA had a greater effect in the Luciferin ME assay, indirubin had a greater effect in the ECOD assay. Luciferin ME assay measures CYP1A2 but not CYP1A1. ECOD activity measures CYP1A1/1A2, in addition to CYP2 and CYP3 activity. Potentially DBA could have a greater effect on CYP1A2 induction, whilst indirubin has a greater effect on CYP1A1 induction; both inducers have been reported by others to induce both CYP1A1 and CYP1A2 expression [61, 136, 207]. Additionally, indirubin could be having a greater effect on other subfamilies measured by the ECOD assay. This was confirmed when the effects of CYP1A inducers on CYP3A function were measured.

As described in Chapter 1, CYP induction arises through nuclear receptor activation and agonists of different nuclear receptors activate various different CYP isoforms. At a

simplistic level, treatment of cells with aryl hydrocarbon receptor (AhR) agonists results in CYP1A activation whereas Pregnane X Receptor (PXR), Constitutive Androstane Receptor (CAR) and Glucocorticoid Receptor (GR) agonists activate CYP2C and CYP3A expression.

In monolayer HepG2 cultures, an effect of AhR agonists on CYP3A induction was observed: indirubin (20µM) increased Luciferin BE metabolism following a 48 hour exposure. There was also a moderate improvement in CYP1A2 function observed following treatment with dexamethasone (50µM) and rifampicin (50µM), although these changes were substantially lower than those measured with DBA and indirubin. In this thesis, the effect of nuclear receptor cross-talk on CYP induction will be addressed in greater detail in Chapter 6.

Cytotoxic effects of each inducer were also measured by WST-1 assay. When normalised to account for total protein content, there was no effect of any of the inducers studied in this assay. It was, however, observed that DBA had a significant effect on cell proliferation, which was reduced following DBA exposure and did not show signs of recovery, even 72 hours after inducer was removed from culture medium. From a regulatory perspective, DBA would not be a preferable inducer for use within a BAL, since it is a polycyclic aromatic hydrocarbon (PAH) and many chemicals of this class are considered carcinogenic. It is also the least soluble of all the inducers selected in this thesis which could lead to difficulties finding a solvent vehicle which is BAL compatible. The effect of DBA on cell proliferation, demonstrated here, provides an additional reason why this chemical, although a powerful inducer of CYP1A enzymes, would not be fit for purpose.

4.2 Cytochrome P450 Function in Encapsulated Hep G2 Cells

Having demonstrated Cytochrome P450 induction in monolayer HepG2 cells, the effects of 3D culture on CYP function in HepG2 cells was then examined.

It has been extensively demonstrated that alginate encapsulation and 3D culture of HepG2 cells can significantly enhance hepatocyte specific functions. Furthermore, bioreactor culture will improve proliferation rates of encapsulated cells; hence large

scale culture becomes feasible. This is, in part, achieved by greater mass transfer which arises through perfusion of cultures by mixing, stirring or rotation.

When alginate encapsulated cells are cultured within a microgravity environment, the resultant decrease in sheer stress and increase in mass transfer that occurs leads to greater cell proliferation and improved viability; consequently the per bead performance is improved [230]. This may also impact on CYP function.

The rotary cell culture system (RCCS) contains a culture module with a gas-permeable central core comprising of a silicone rubber membrane. The module, which is filled with beads and medium, constantly rotates at a slow speed (~8rpm) on a horizontal axis so that the beads are in constant free-fall but held in suspended animation by the rotational drag of the medium (Figure 4-7).

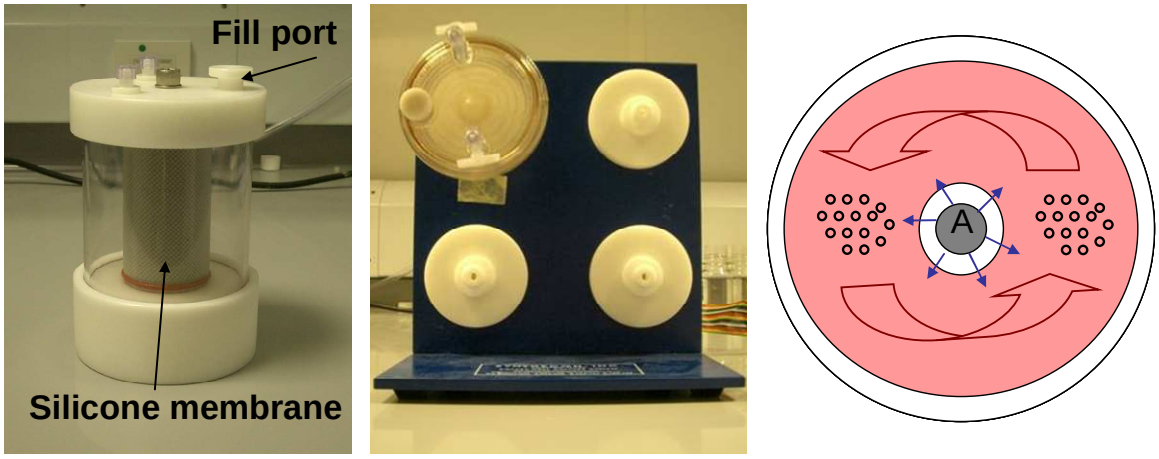


Figure 4-7. Setup of the RCCS. Beads and culture medium are added to the culture module (left) via a fill port which is then sealed. Up to 4 culture vessels can be screwed onto a base (middle) which is placed into a 37°C incubator and powered by a motor to rotate the vessels at 8rpm. The illustration of a cross section of an RCCS vessel (right) demonstrates how mass transfer occurs: each vessel is rotated on a horizontal axis (A) allowing culture medium to circulate and perfuse the beads which are maintained in suspension. Gas exchange (→) occurs through a central core which is gas permeable.

As described above, the RCCS is a sealed culture system which provides a perfused mass transfer system by rotating. In contrast, the fluidised bed bioreactor (FBB) consists of a perfusable culture chamber associated with a large medium reservoir. Fluidisation is achieved by pumping medium from the reservoir into the base of the culture chamber. Beads under fluidised conditions move in a loop from the bottom of the chamber to maximum fluidisation height due to the opposing forces of gravity acting on the beads, which are denser than the medium, and upward drag created by the flow of medium through the bioreactor (Figure 4-8). Temperature, pH and oxygen concentration are all controlled by a fermentor.

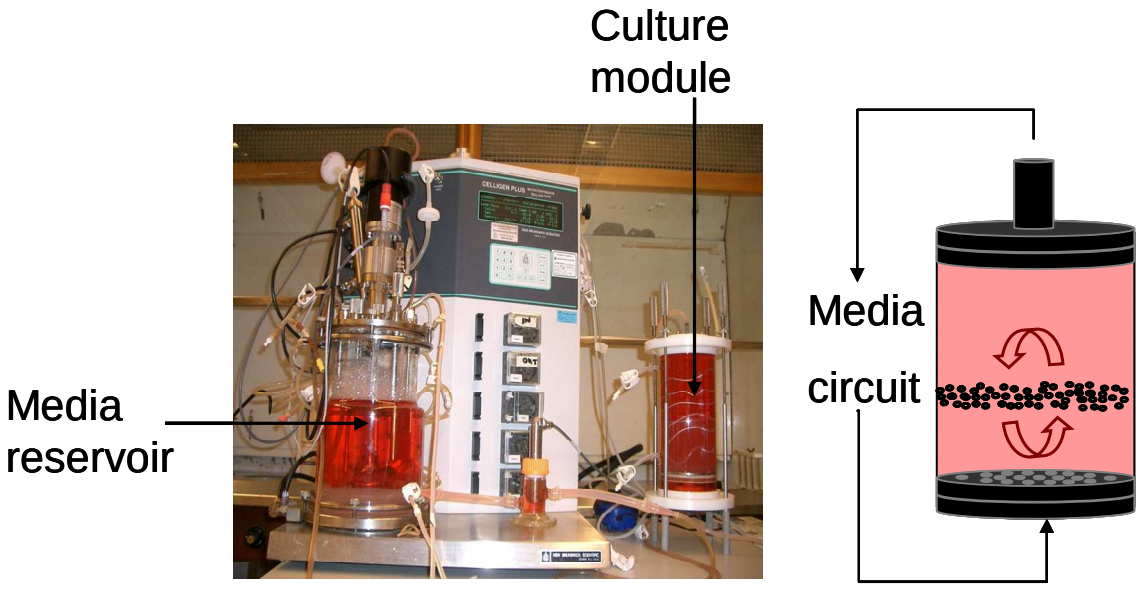


Figure 4-8. Setup of the FBB culture system. Beads are maintained within a culture module which is continually perfused with medium held in a separate reservoir. Temperature, pH and oxygenation are tightly regulated by a fermentor. Media is pumped at a controlled rate through the culture module from the base to the top by a peristaltic pump. The flow of media causes the beads to rise and gravity then causes the beads to fall again meaning they move in a continual loop around the bioreactor.

In the following section, Cytochrome P450 function was examined in encapsulated HepG2 cells maintained in static culture or within a microgravity/fluidised bed environment.

4.2.1 Methods

4.2.1.1 CYP Function in Encapsulated HepG2 Cells Maintained in Static Culture

HepG2 cells were encapsulated using the Inotech method described in Chapter 2 and beads resuspended in HG αMEM (Chapter 2) with 10% FCS at a ratio of 0.25ml of beads per 8ml of media. 8ml/well of this suspension was added to 6 well plates and beads maintained in static culture with media changes every 2 days. Inducers were added on day 6 for 48 hours. CYP function was measured on day 8 by ECOD, Luciferin BE or TST assay.

4.2.1.2 CYP Function in HepG2 Cells Maintained in Microgravity Culture

HepG2 cells were encapsulated using the Jetcutter method described in Chapter 2 and beads resuspended in HG αMEM with 10% FCS at a ratio of 0.25ml beads per 8ml media. Beads were added via a fill port to RCCS chambers (500mls of suspension (16ml beads)/vessel) which were screwed onto the RCCS base and then placed within a 37°C humidified incubator (95% air, 5% CO2). Beads were maintained in culture for up to 10 days and 50% (250mls) of the culture medium was replenished every 2 days. Inducers were added on day 6 for 48 hours and CYP activity was then measured by ECOD or Luciferin BE assay.

4.2.1.3 CYP Function in HLM Isolated From HepG2 Cells Following Bioreactor Culture

As described, during FBB culture, conditions including pH and dissolved oxygen concentration are tightly regulated. The very high cell density achieved by this culture system means that assessing CYP function in these encapsulated cells under standard assay conditions would have impact on cell function and viability. Therefore, once HepG2 cells were removed from the FBB, human liver microsomes (HLM) were isolated and these were used for measurement of CYP activity. CYP function was compared in HLM prepared from freshly isolated primary human hepatocytes (HLM-PHH) from 5 separate donors (PHH3, PHH8, PHH25, PHH28 and PHH31) or HepG2 cells (HLM-HepG2). HepG2 cells were in their basal state (uninduced) and had either been grown in an RCCS for 8 days (section 4.2.1.2), or were obtained following propagation in a fluidised bed bioreactor (FBB).

4.2.1.3.1 FBB Culture of HepG2 Cells

The overall aim of FBB experiments is to optimise large scale culture conditions for HepG2 cells in order to achieve maximum bead capacity without adversely affecting cell function. This work was performed by others who provided the alginate beads used here.

HepG2 cells were encapsulated at an initial seeding density of $1.5x10^6$ cells/ml using the Jetcutter method described in Chapter 2. A total volume of 200mls of alginate beads were encapsulated and these were resuspended in HG αMEM containing 10% FFP and then added to a 2.5L (15cm diameter) FBB associated with a 7.5L circuit. Medium was perfused through the circuit at 390ml/minute to achieve 1.5 times fluidisation height. Beads were maintained at 37°C; temperature, pH and oxygen supply were tightly regulated. 50% medium changes were carried out at d2, d5, d7, d9 and d11. A 25% medium change was performed at d12. Beads were harvested on d13 at which point cell viability was determined by FDA/PI staining to be 98%.

4.2.1.3.2 HLM Isolation and Measurement of CYP Function

HepG2 spheroids were released from alginate using the method described in Chapter 2. HLM were isolated from these or PHH and CYP function quantified (ECOD or Luciferin BE assay) using the methods described in Chapter 3 (section 3.1.2).

4.2.2 Results

Cytochrome P450 function was firstly measured in encapsulated HepG2 cells grown in static culture in 6 well plates. Subsequently, CYP function was compared in encapsulated HepG2 cells maintained in microgravity/FBB culture systems.

4.2.2.1 3D Culture of HepG2 Cells Maintained in Static Culture

Figure 4-9 shows the growth and viability of encapsulated cells maintained in static culture. FDA (live cell) staining demonstrates proliferation and spheroid formation throughout 8 days of culture. PI (dead cell) staining indicates that at day 8, when cell density in the beads was highest, there was a slight decline in viability.

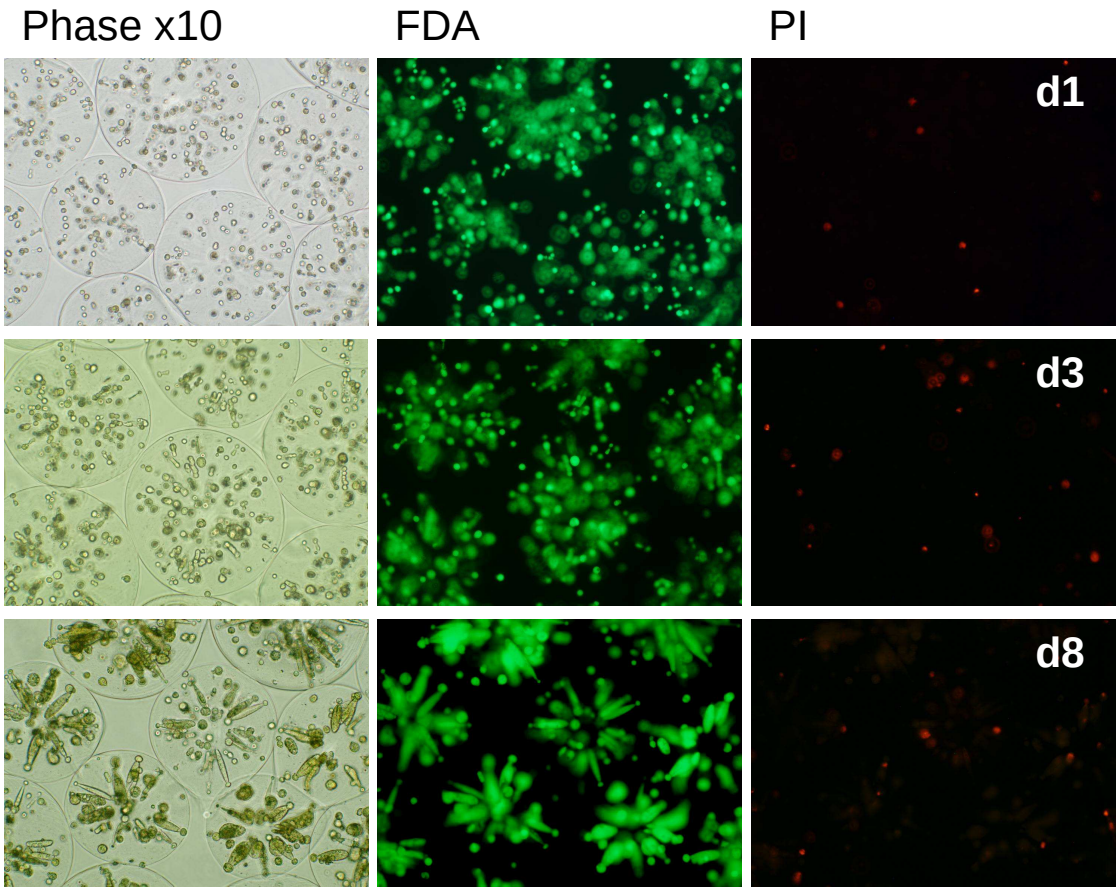


Figure 4-9. Cell viability staining (x10 magnification) of encapsulated HepG2 cells maintained for up to 8 days in static culture.

4.2.2.1.1 ECOD Activity in Encapsulated HepG2 cells Maintained in Static Culture

ECOD activity was compared in monolayer and 3D cultures of HepG2 cells, following 48 hour DBA induction, and improved function was observed after 3D culture.

Data was analysed by two-way ANOVA and is shown in Figure 4-10. There was a significant effect of 3D culture on ECOD activity in HepG2 cells ($P<0.05$, $F(1, 0.0025) = 11.26$) and a significant effect of DBA induction in these cultures ($P<0.01$, $F(1, 0.0047) = 21.20$) however there was no synergism between the two factors.

ECOD activity was elevated in cells maintained in 3D culture following treatment with 0.1% DMSO vehicle (2.6 fold above monolayer culture) and 10µM DBA (1.8 fold).

In both culture formats, DBA resulted in induction of ECOD activity. Extent of induction was slightly higher in monolayer cultures (3.3 fold induction above basal) than in 3D cultures (2.3 fold) but this difference was not significant.

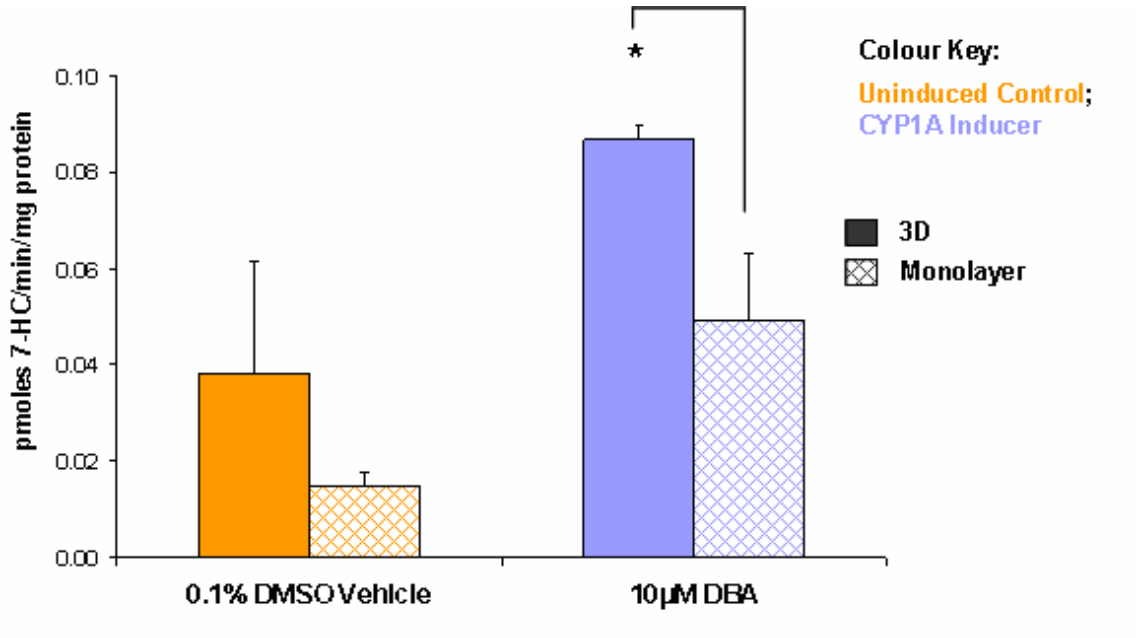


Figure 4-10. Comparison of ECOD activity in HepG2 cells cultured in 3D format for 8 days or in monolayer and treated for 48 hours with either 0.1% DMSO vehicle or 10µM DBA (n=3 +/- sd). Data analysis was by two-way ANOVA with Bonferroni post test. * P <0.05 when compared to monolayer cultures.

4.2.2.1.2 CYP3A (Luciferin BE) Activity in Encapsulated HepG2 Cells Maintained in Static Culture

Luciferin BE metabolism was compared in HepG2 cells maintained in monolayer or 3D culture and treated for 48 hours with 50µM rifampicin or 0.1% DMSO vehicle. Improved function was observed after 3D culture (Figure 4-11).

There was a significant effect of 3D culture on CYP3A activity in HepG2 cells ($P<0.001$ $F(1, 0.001) = 53.54$) and significant interaction between effects of rifampicin and effects of 3D culture ($P < 0.01$ $F(1, 0.0001) = 8.61$). Under basal conditions, 3D culture resulted in an increase in CYP3A activity (2.8 fold). However, the effect of rifampicin induction in 3D cultured cells was lost; the difference in function between 3D and monolayer cultures was therefore decreased (1.4 fold difference) following rifampicin treatment.

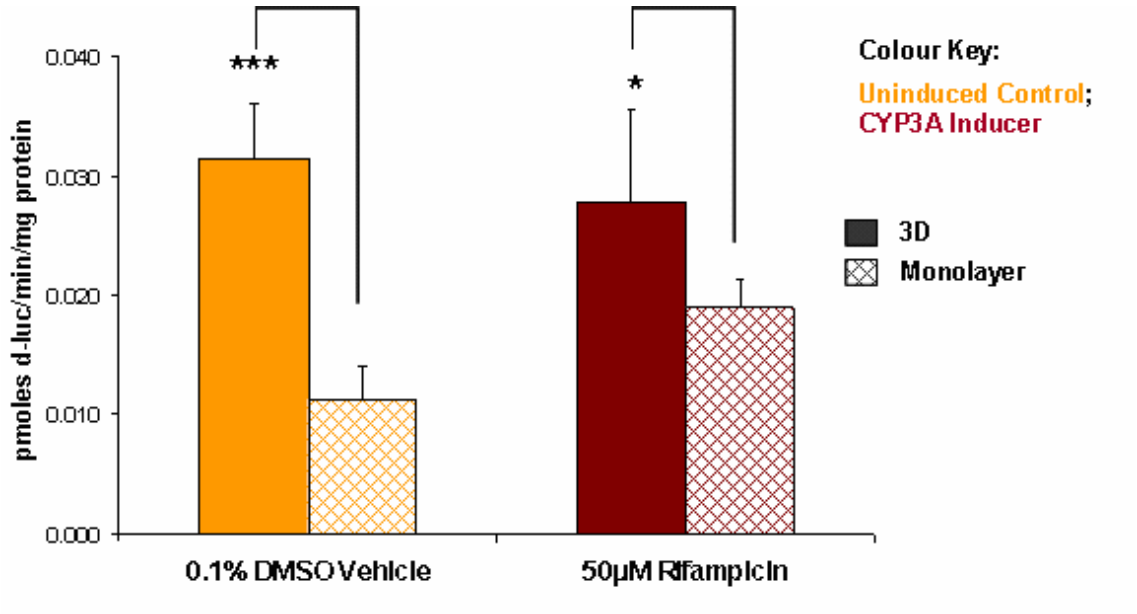


Figure 4-11. CYP3A function was measured in HepG2 cells cultured in 3D format for 8 days or in monolayer and treated for 48 hours with either 0.1% DMSO vehicle or 50µM rifampicin (n=6 +/- sd). Data analysis was by two-way ANOVA with Bonferroni post test. * P <0.05, *** P < 0.001 when compared to monolayer cultures.

4.2.2.1.3 Testosterone Metabolism in Encapsulated HepG2 Cells

Substrate metabolism was analysed in monolayer or encapsulated HepG2 cells that had been treated for 48 hours with 50µM rifampicin and incubated for up to 8 hours with 200µM testosterone (TST). Included in the same analysis run was supernatant from freshly isolated PHH treated for 1 hour with 200µM TST.

After 1 hour incubation with TST, encapsulated HepG2 cells did not produce any detectable metabolite of the substrate (Figure 4-12).

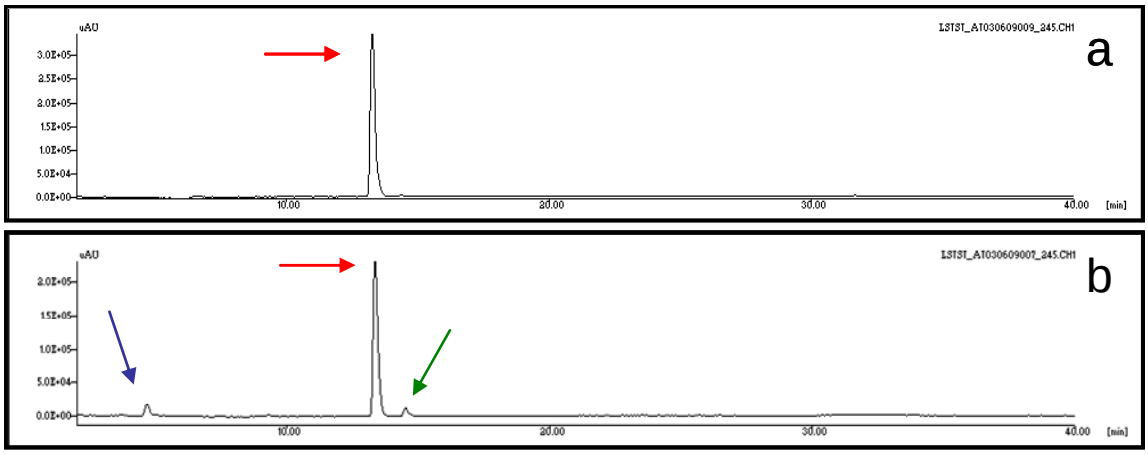


Figure 4-12. HPLC was used to measure metabolism of testosterone in (a) encapsulated HepG2 cells treated with 50µM rifampicin or (b) freshly isolated PHH incubated for 1 hour with 200µM TST. TST spike is indicated by →. TST metabolites are indicated by →/→ and were measured in PHH only. One representative trace is shown of 4 independent measurements.

Following an 8 hour incubation with TST, HepG2 cells had produced a similar metabolite to that produced by PHH following 1 hour substrate incubation. Substrate metabolism was also compared with monolayer HepG2 cells and appeared greater in 3D culture (Figure 4-13).

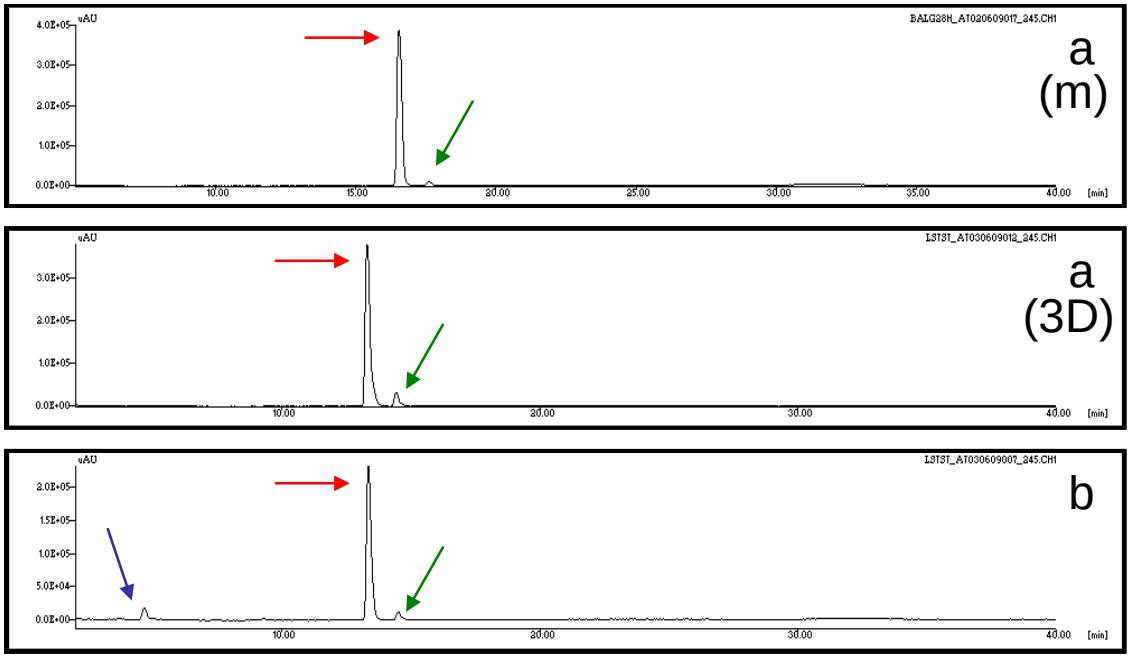


Figure 4-13. HPLC was used to measure metabolism of testosterone in (a) HepG2 cells treated with 50µM rifampicin in monolayer (m) or 3D culture and then incubated with 200µM TST for 8 hours, or (b) freshly isolated PHH incubated with 200µM TST for 1 hour. TST spike is indicated by →. TST metabolites are indicated by →/→ and differed in PHH and HepG2 cells. One representative trace is shown of 4 independent measurements.

4.2.2.2 3D Culture of Encapsulated HepG2 Cells Maintained in Microgravity Culture

Figure 4-14 shows the growth and viability of encapsulated cells maintained for 8 days within an RCCS microgravity environment. FDA staining demonstrates proliferation and spheroid following 8 days of culture. There was minimal PI staining which demonstrates that viability was improved with this culture system and was maintained even when bead density increased.

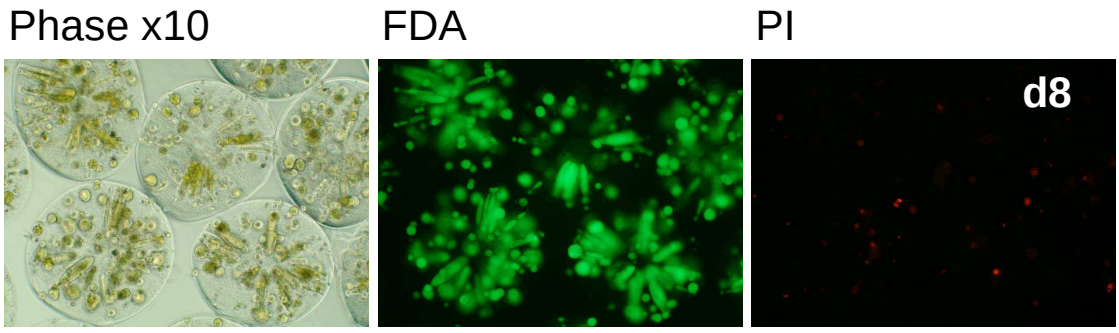


Figure 4-14. Cell viability staining (x10 magnification) of encapsulated HepG2 cells following 8 days of RCCS microgravity culture.

4.2.2.2.1 ECOD Activity in HepG2 Cells Following RCCS Culture

ECOD activity was compared in monolayer cultures and in 3D cultures of HepG2 cells following either static or microgravity culture. Improved induction was observed following microgravity culture (Figure 4-15, note that, in this figure, activity is expressed relative to that seen in monolayer culture).

Data was analysed by two-way ANOVA. There was a significant effect of culture format on ECOD activity in HepG2 cells ($P<0.001$, $F(3, 0.143) = 377$) and a significant effect of DBA induction in these cultures ($P<0.001$, $F(1, 0.106) = 561$). There was also a significant interaction between the two factors ($P<0.001$, $F(2, 0.123) = 322$); DBA mediated induction was enhanced in cells maintained in RCCS environment.

Under basal conditions, ECOD activity was increased by equivalent amounts following static (2.6 fold above monolayer) or RCCS (2.6 fold) culture. Following DBA exposure, a greater increase was seen in the RCCS (8.8 fold above monolayer) than in static culture (1.8 fold above monolayer). Therefore the extent of DBA mediated induction

was greater following RCCS culture (11.4 fold induction above basal), than in static (2.3 fold induction), or monolayer culture (3.3 fold induction).

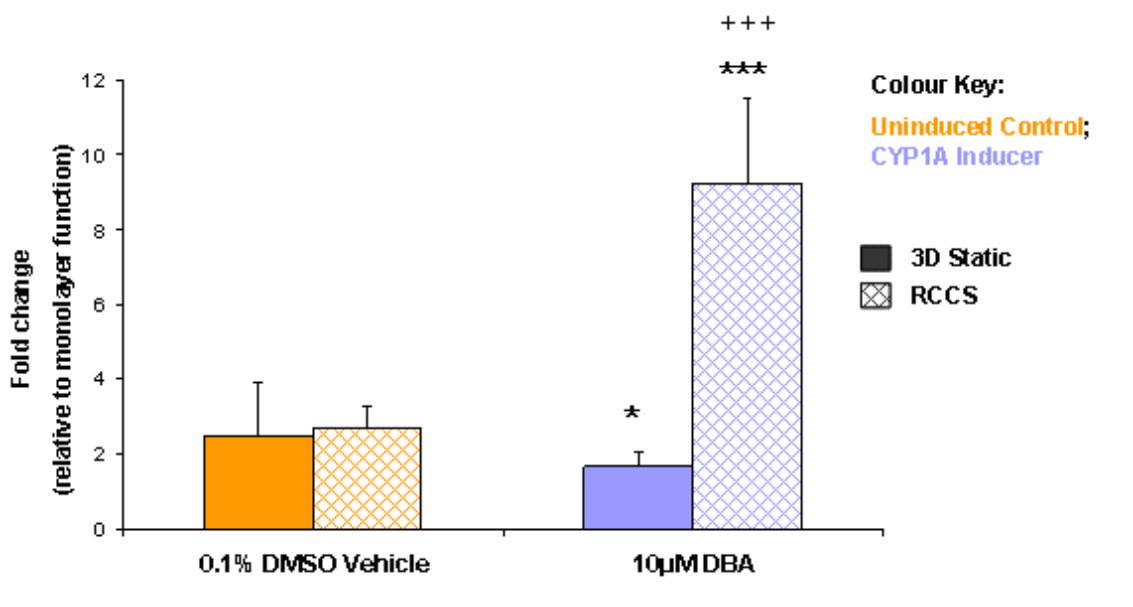


Figure 4-15. ECOD activity was measured in HepG2 cells cultured in 3D format maintained for 8 days in either static or microgravity culture and treated for 48 hours with either 0.1% DMSO vehicle or 10µM DBA. For each culture system, change in activity relative to monolayer culture was calculated and is expressed here as mean (n=3) +/-sd. Data analysis was by two-way ANOVA and Bonferroni post test. * P <0.05, *** P<0.001 when compared to monolayer cultures.

+++ P < 0.001 when static and RCCS cultures are compared.

4.2.2.2.2 CYP3A (Luciferin BE) Activity in HepG2 Cells Following Microgravity Culture

CYP3A (Luciferin BE) activity in monolayer cultures of HepG2 cells under basal conditions were compared with 3D cultures maintained in either static or microgravity culture. 3D culture resulted in improved CYP3A activity; the greatest improvement in function was observed when encapsulated cells were maintained in an RCCS environment (Figure 4-16).

Data was analysed by one-way ANOVA. There was a significant difference in Luciferin BE metabolism between culture systems (P<0.001). CYP3A activity was significantly increased in 3D culture when cells were maintained in static culture (P<0.01, 2.8 fold above monolayer). Function was further increased by RCCS culture of encapsulated cells (P<0.001, 3.8 fold above monolayer).

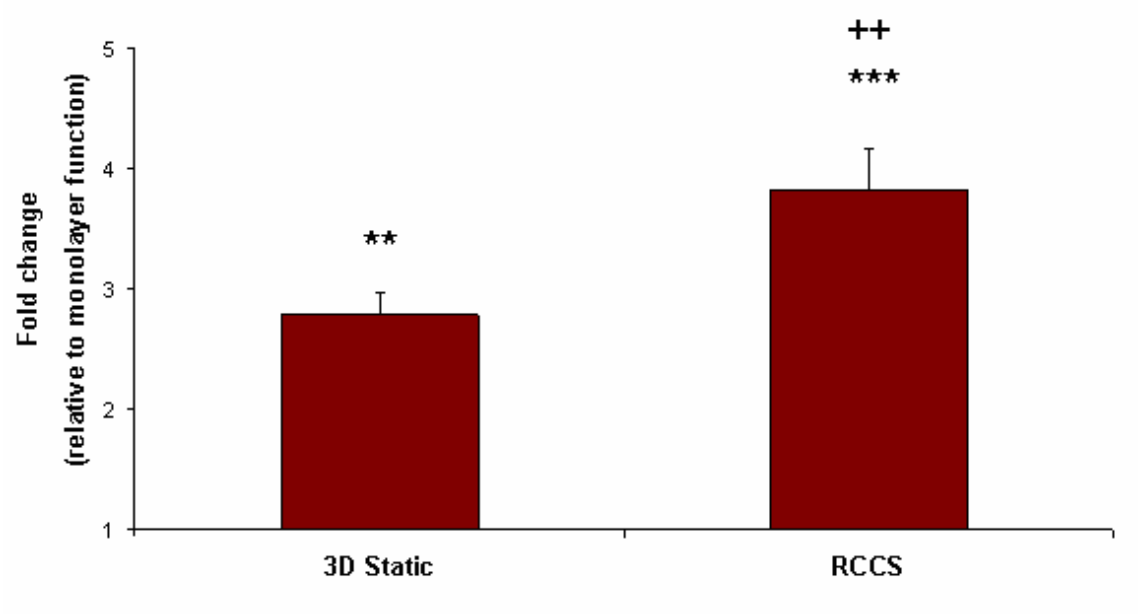


Figure 4-16. CYP3A (Luciferin BE) activity was measured in HepG2 cells cultured in 3D format maintained for 8 days in either static or microgravity culture. For each culture system, change in activity relative to monolayer culture was calculated and is expressed here as mean (n=3) +/-sd. Data analysis was by one-way ANOVA and Bonferroni post test. ** P < 0.01, *** P < 0.001 when compared to monolayer culture.

++ P < 0.001 when static and RCCS cultures are compared.

4.2.2.2.3 CYP Activity in HLM isolated from HepG2 Cells Following Bioreactor Culture

CYP function was compared in HLM isolated from freshly isolated PHH and from HepG2 cells maintained in two different bioreactor systems (RCCS or FBB) in either 10% FCS or 10% FFP respectively (Figure 4-17). PHH were isolated from 5 separate donors and activity in each HLM preparation was measured separately (as opposed to pooling donor samples). This was done so that both mean and range of activity for PHH from separate donors could be calculated.

The level of ECOD activity in HLM-HepG2 was comparable between the two bioreactor systems. ECOD activity in HLM-HepG2 approached the lower levels of function measured in HLM-PHH but was substantially lower than the average value calculated for all 5 donors (5.0 - 6.6 fold less than HLM-PHH for FBB and RCCS HLM-HepG2).

CYP3A (Luciferin BE) activity in HLM-HepG2 from RCCS culture was only slightly lower (1.3 fold) than the average value measured in HLM-PHH but was considerably higher (4 fold) than HLM-HepG2 obtained following FBB culture.

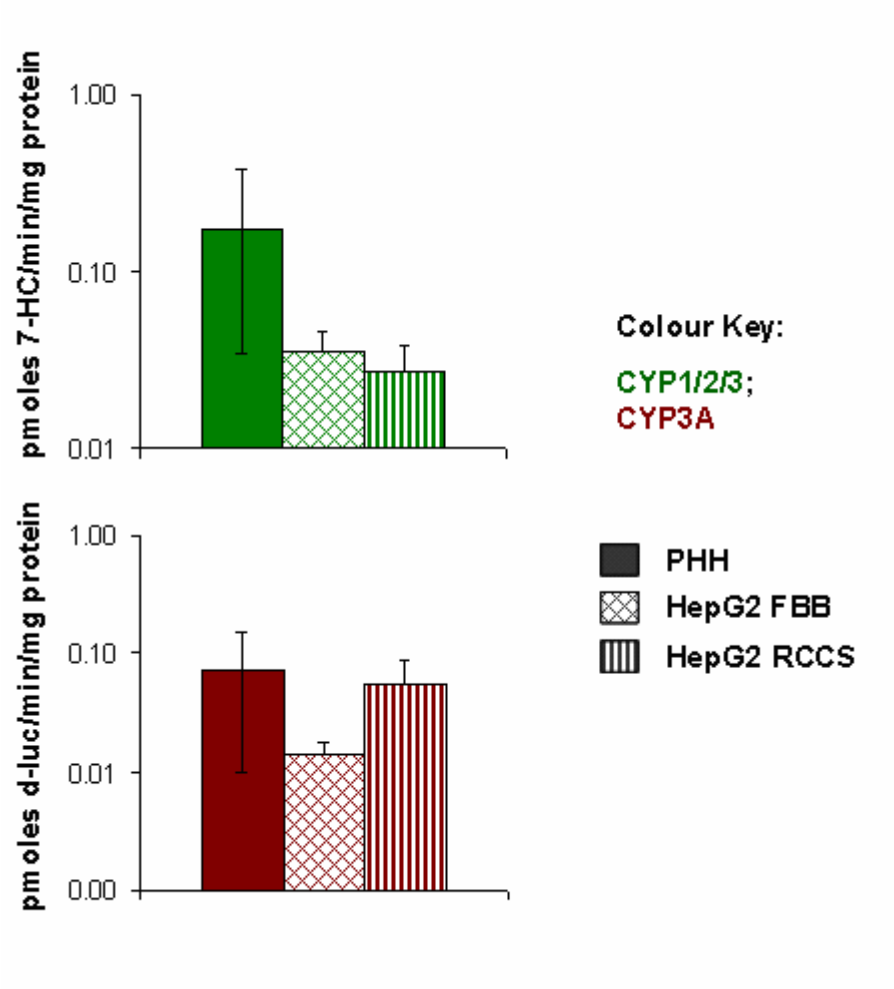


Figure 4-17. Cytochrome P450 function was measured by (top) ECOD assay and (bottom) Luciferin BE assay to compare activity in HLM isolated from freshly isolated PHH and 3D bioreactor cultures of HepG2 cells maintained in an RCCS in 10% FCS or an FBB in 10% FFP. Mean and range of activity is shown for PHH isolated from 5 separate donors (measured in triplicate/donor). HepG2 data is expressed as mean (n=2) +/-range. Note the logarithmic scale of the *y* axis.

4.2.3 Discussion

HepG2 encapsulation and subsequent 3D culture resulted in a significant improvement in Cytochrome P450 function. ECOD activity was improved under basal conditions and following DBA induction, although extent of induction was slightly less in 3D cultures maintained in static culture. When static and microgravity cultures were compared, under basal conditions CYP function was comparable, however, DBA mediated induction was significantly improved following culture in an RCCS environment. This could be partly attributed to the increased viability of cells in microgravity culture since ECOD data was normalised to total protein rather than viable cell number. The observed effect in monolayer culture of DBA on cell proliferation could also have an effect.

Under basal conditions, CYP3A activity was significantly improved by 3D culture; microgravity culture also resulted in further improvement in function relative to static culture. Once again this can probably be related to improved cell viability and cell density, and hence per bead performance, that is observed following microgravity culture. The effect of rifampicin mediated induction on CYP3A activity was also compared in monolayer and 3D (static) cultures and it was demonstrated that the effect of induction was reduced by 3D culture. This indicates that the extent of induction that can be achieved in HepG2 cells is limited. As described previously, CYP3A induction is governed by nuclear receptors including PXR and CAR and co-receptor RXRα. Others have reported diminished nuclear receptor expression in hepatoma cell lines [157, 221, 232]; potentially this could have a limiting effect on CYP3A induction in these cell lines in spite of the overall positive effect of 3D culture.

Improved Luciferin BE metabolism in 3D culture indicates that CYP3A function was improved. Testosterone metabolism was also measured in encapsulated HepG2 cells. HepG2 cells were incubated with testosterone for up to 8 hours and after this time although they appeared to metabolise testosterone they did not produce the CYP3A4 metabolite 6β-OHTST. Possibly alternative isoforms, including CYP3A7, were responsible for substrate metabolism and these were upregulated by 3D culture. This will be discussed further in Chapters 5 and 6.

HLM were prepared from HepG2 cells maintained in their basal state in two different bioreactor systems: RCCS and FBB. Cytochrome P450 activity was compared between the two systems and also in HLM prepared from freshly isolated PHH.

ECOD activity was comparable between the two bioreactor systems (without induction) and was approaching lower levels measured in PHH. This is in contrast to monolayer culture whereby lower levels of PHH activity were only matched in HepG2 cells following DBA or indirubin induction.

CYP3A (Luciferin BE) activity in HLM isolated from HepG2 cells following bioreactor culture matched levels measured in HLM from freshly isolated PHH, however, there was a difference in CYP3A function between the two bioreactor systems: CYP3A activity was decreased in HepG2 following FBB culture. Several differences in bioreactor setup could account for this variation.

Whereas RCCS experiments were performed in an optimised culture system, the culture of beads in the FBB experiment was performed by others, with the aim optimising conditions for cell growth within a bioreactor, and experiment parameters were therefore different. Firstly, beads cultured in the RCCS were harvested following 8 days of culture, whereas beads in the FBB were maintained for 13 days, and it has been shown that many cell functions in HepG2 spheroids are optimal following 8 days of static culture [109]. Secondly, the RCCS is a sealed system in which pH and temperature are regulated by a 37°C 5% CO_2 incubator, whereas the FBB is continually perfused with medium whose temperature, pH and oxygen content is tightly regulated by a fermentor; oxygen delivery in particular may have an effect on HepG2 CYP function. In hepatocytes, CYP enzymes are preferentially expressed in the perivenous zone, where oxygen concentration is lower and others have shown that CYP1A activity measured in HepG2 was decreased in hyperoxic conditions [5]. Most importantly, beads maintained in the FBB were cultured in 10% FFP rather than 10% FCS. Plasma, as well as the heparin which was used as an anticoagulant, may have had an effect on CYP function and this will be explored below.

4.3 Effects of Human Plasma Exposure on HepG2 cells

A beneficial effect of FFP exposure on HepG2 proliferation has been observed when cells are cultured within the LG Fluidised Bed Bioreactor (FBB); potentially, CYP expression could be affected by plasma and so the influence of human FFP on CYP induction was investigated. Furthermore, it was observed that HLM prepared from HepG2 cells cultured in FFP (section 4.2.1.3.1) produced a greater quantity of fat during the isolation process. Possibly, exposure of HepG2 cells to FFP could render them steatotic and this could have an effect on CYP function [64, 65]. Fat loading was therefore investigated by labelling cells with Nile red, which is a lipophilic dye, which fluoresces strongly when partitioned into lipids, but not in aqueous solution [233].

4.3.1 Methods

Human FFP obtained from multiple donors was prepared as described in Chapter 2. FCS containing 40 IU/ml heparin was also included in these experiments as a control.

4.3.1.1 Monolayer Induction

HepG2 Cells were seeded in 96 well plates at a density of $2.5x10^4$ cells/well in a volume of 100µl of complete culture medium containing 10% of FCS +/- heparin or FFP. Following overnight attachment, cells were treated with CYP inducers for 48 hours and then CYP activity measured by ECOD assay, Luciferin BE assay or Luciferin ME assay (+ 10µM sulfaphenazole). Cell protein was determined by the BCA assay.

4.3.1.2 3D-Culture and P450 Induction of FFP Exposed Cells

Cells were encapsulated using the Inotech method. Beads were resuspended in HG αMEM containing either 10% FCS + heparin or FFP. Medium was also supplemented with an additional amount of calcium chloride (200µM) to account for any further effects of calcium sequestration by FFP which would result in reversal of alginate polymerisation. Beads were maintained in static culture, as described for Chapter 2. Inducers were added for 48 hours on day 6 and, following induction, CYP activity was measured by ECOD assay. Spheroids were then released from alginate (Chapter 2) and protein determined by the BCA method.

4.3.1.3 Nile Red Staining of HepG2 Cells

Nile red is a phenoxazone dye which was used as a fluorescent hydrophobic probe to measure lipid accumulation in HepG2 cells [234] following FFP exposure.

A 2mg/ml solution of Nile Red (Sigma N3013) was prepared in methanol. This was diluted to 5µg/ml in unsupplemented phenol red free DMEM.

HepG2 cells were treated, as described above for monolayer culture, with CYP inducers or 0.1% DMSO (vehicle control) for 48 hours. Cells were then washed twice with PBS and 200µl of the diluted Nile red solution added. Cells were incubated at 37°C for 2 hours with the dye and then washed twice again with PBS. Cells were incubated with phenol red-free DMEM for a further 30 minutes and visualised using a fluorescence microscope with a FITC filter (excitation 465-495 nm/ emission 515-555 nm). Nile red straining was quantified by reading plates using a cytofluor (excitation 485 nm emission 530nm).

4.3.2 Results

CYP function was compared in HepG2 cells induced in αMEM complete containing either 10% FCS or 10% FFP and data was analysed by two-way ANOVA.

4.3.2.1 Effect of FFP on ECOD Activity in Monolayer HepG2 Cultures

Increased ECOD activity was observed following FFP exposure. This effect was inducer dependent and there was a slight influence of heparin in some instances. This data is shown in Figure 4-18.

ECOD activity following induction was compared between cultures maintained in FCS +/- heparin and FFP. There was a significant effect of both FFP exposure ($P<0.05$, $F(2, 0.481) = 5.23$) and chemical induction ($P < 0.01$, $F(4, 0.216) = 11.77$) on CYP function.

Under basal conditions, ECOD activity in FCS cultures +/- heparin was comparable. FFP exposure led to a slight (1.3 fold above FCS function) increase in ECOD activity. This was further increased by rifampicin (1.7 fold) and DBA induction ($P < 0.05$, 1.8 fold).

Following exposure to heparin, CYP induction was increased, relative to cells cultured in 10% FCS alone, following treatment with indirubin (1.5 fold) and dexamethasone (1.4 fold). FFP had no further effect on cells treated with indirubin, however, dexamethasone induction was further increased (2.0 fold above FCS function) in cells cultured in 10% FFP.

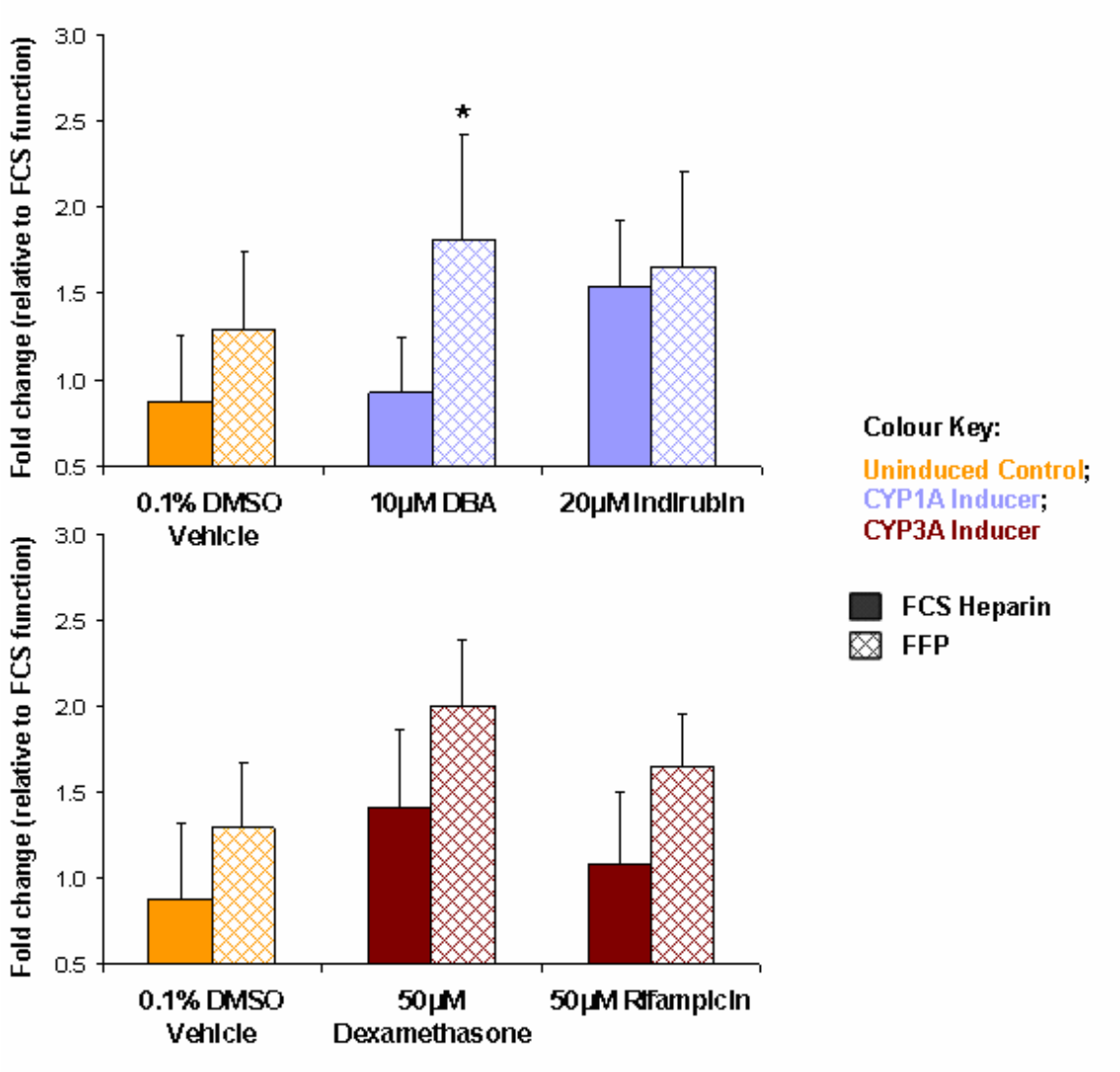


Figure 4-18. Effect of CYP induction (48 hours) on ECOD activity was measured in HepG2 cells following monolayer culture in complete culture medium supplemented with 10% FCS +/- heparin or 10% FFP. For each inducer, change in function relative to standard culture conditions (αMEM complete 10% FCS) was calculated and is expressed as mean (n=3) +/-sd. Data analysis was by two-way ANOVA and Bonferroni post test. * P < 0.05 when compared to FCS culture.

4.3.2.2 *Effect of FFP on CYP1A2 (Luciferin ME) Activity in Monolayer HepG2 Cultures*

Luciferin ME metabolism was measured in the presence of 10µM sulfaphenazole. In this assay there was no effect of heparin or FFP exposure on CYP function in either basal conditions or in the presence of inducer (Figure 4-19).

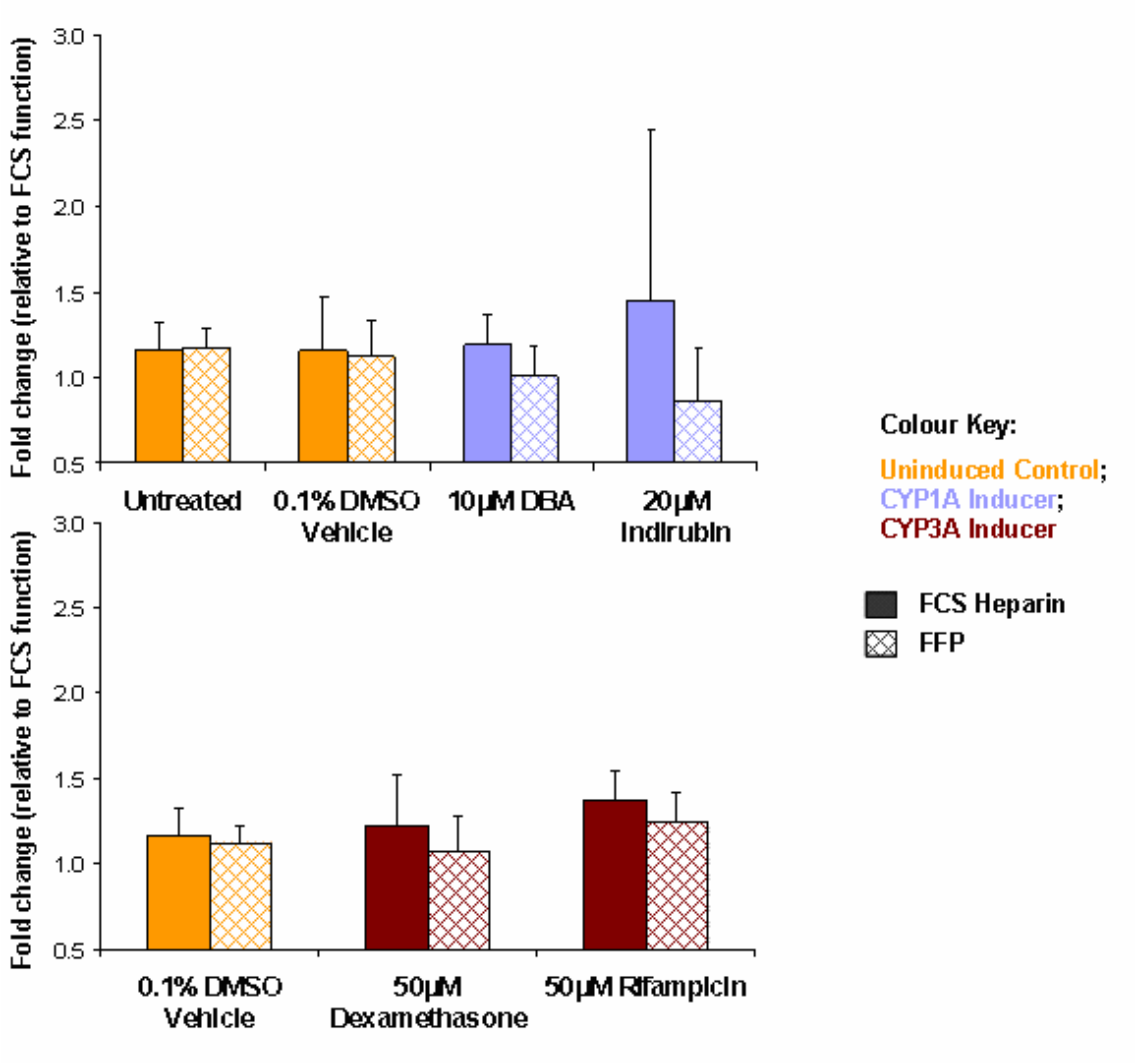


Figure 4-19. Effect of CYP induction (48 hours) on CYP1A2 activity (Luciferin ME metabolism detected in the presence of 10µM sulfaphenazole) was measured in HepG2 cells following monolayer culture in complete culture medium supplemented with 10% FCS +/- heparin or 10% FFP. For each inducer, change in function relative to standard culture conditions (αMEM complete 10% FCS) was calculated and is expressed as mean (n=3) +/-sd.

4.3.2.3 Effect of FFP on CYP3A (Luciferin BE) Activity in Monolayer HepG2 Cultures

FFP exposure had a negative effect on CYP3A function; however this was lessened by rifampicin mediated induction. Data was analysed by two-way ANOVA and is presented in Figure 4-20.

There was a significant effect of FFP exposure ($P < 0.001$ $F(2, 0.924) = 35.22$) and induction ($P < 0.001$, $F(5, 1.325) = 18.79$) on CYP3A activity in HepG2 cells,

CYP induction was comparable in cells cultured in either 10% FCS or 10% FCS + heparin, however exposure to 10% FFP resulted in decreased CYP3A activity. Moreover, FFP mediated decreases in CYP3A function were further affected by induction. This was particularly apparent for cells treated with CYP1A inducers DBA ($P<0.05$ 0.71 fold of FCS function) and indirubin ($P < 0.05$, 0.60 fold of FCS function); positive effects of these inducers on CYP3A function (described in section 4.1.2.2) were diminished following FFP exposure.

The negative effect of FFP exposure on Luciferin BE metabolism was improved to a certain extent by rifampicin treatment; although still lower than measured in FCS cultures, CYP3A activity was increased (0.85 fold of FCS function) in cells cultured in 10% FFP and treated with rifampicin, thus the effect of this inducer was maintained in the presence of FFP.

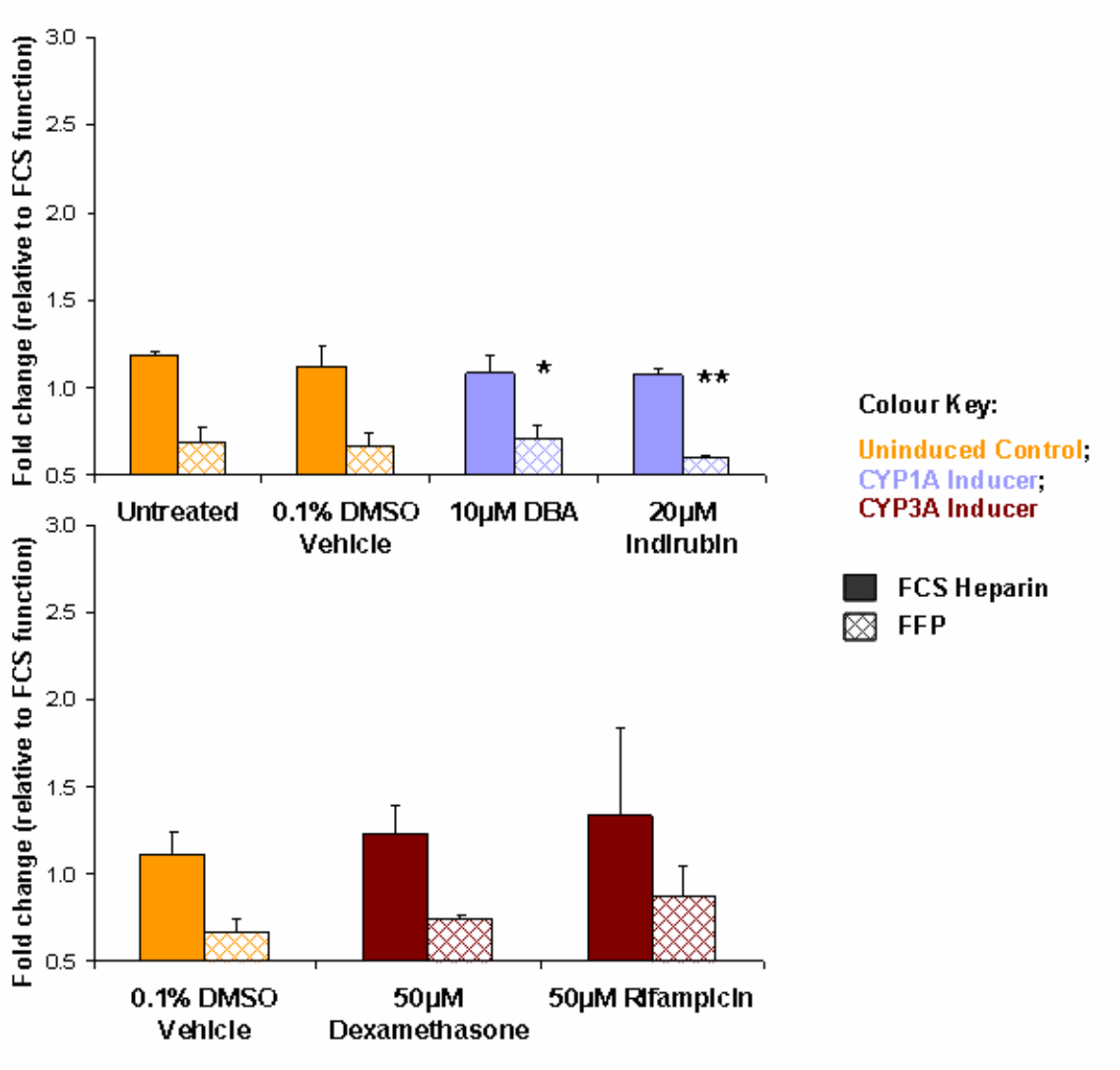


Figure 4-20. Effect of CYP induction (48 hours) on CYP3A activity (Luciferin BE metabolism) was measured in HepG2 cells following monolayer culture in complete culture medium supplemented with 10% FCS +/- heparin or 10% FFP. For each inducer, change in function relative to standard culture conditions (αMEM complete 10% FCS) was calculated and is expressed as mean (n=3) +/- sd. Data analysis was by two-way ANOVA and Bonferroni post test. * P < 0.05, ** P<0.01 when compared to FCS culture.

4.3.2.4 Effect of 3D Culture on CYP Function Following FFP Exposure

ECOD activity was measured in encapsulated cells induced for 48 hours in medium containing either 10% FCS or 10 % FFP (both treated with 40IU/ml heparin). For each condition function was compared to the corresponding monolayer culture.

Under basal conditions, ECOD activity was increased (1.59 fold above monolayer) relative to monolayer culture in cultures exposed to 10% FCS + heparin, but was decreased (0.8 fold of monolayer activity) in cultures exposed to 10% FFP.
The effect of 3D culture on ECOD activity differed for each inducer, however, these effects were comparable following exposure to either FCS or FFP; there were no statistically significant differences between the two media supplements. This data is shown in Figure 4-21 and is summarised below:

10µM DBA (CYP1A inducer): as described in section 4.2.2.1.1, extent of DBA induction was slightly reduced in 3D culture. Following DBA exposure, ECOD activity was increased 1.29 fold above monolayer cultures in cells cultured in 10% FCS and was decreased (0.80 of monolayer culture) in cells cultured in 10% FFP.

20µM Indirubin (CYP1A inducer): induction was increased in 3D culture. Following indirubin exposure, ECOD activity was increased 2.3/3.0 fold above monolayer cultures in cells cultured in 10% FCS/FFP.

50µM Dexamethasone (CYP3A inducer): exposure resulted in decreased activity in 3D culture. ECOD activity following treatment with dexamethasone was decreased in both FCS (0.17 fold of monolayer activity) and FFP (0.30 fold) cultures.

50µM Rifampicin (CYP3A inducer): induction was increased in 3D culture. Following rifampicin exposure, ECOD activity was increased 2.6/1.6 fold above monolayer cultures in cells cultured in 10% FCS/FFP.

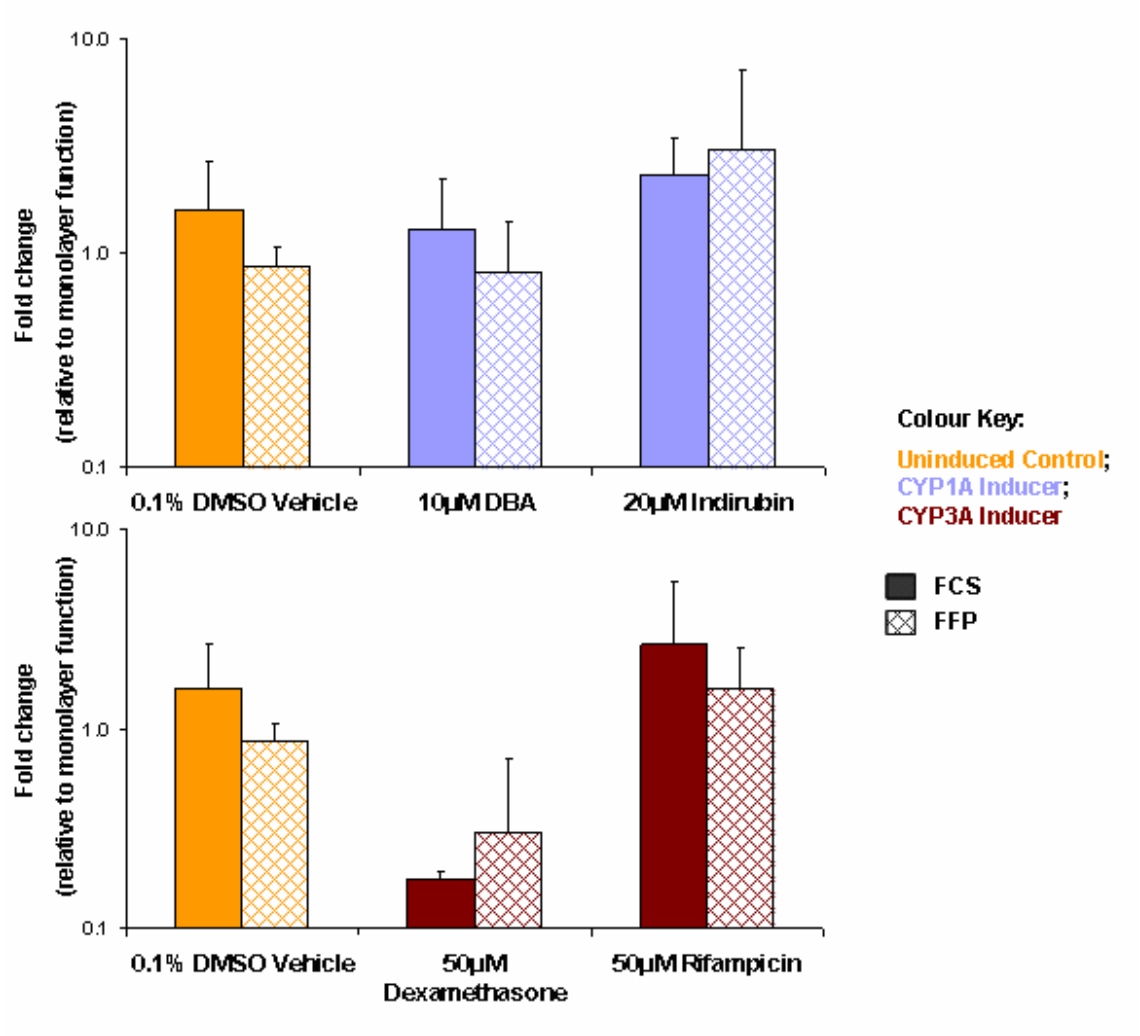


Figure 4-21. ECOD activity following treatment with CYP inducers was compared in monolayer and 3D cultures of HepG2 cells cultured in either 10 % FCS with heparin or 10% FFP. For each inducer fold difference between 3D and monolayer function was calculated and data is expressed as mean (n=3) +/- sd.

4.3.2.5 Effect of FFP on Nile Red Staining

The effect of FFP on Nile red staining in HepG2 cells is shown in Figure 4-22. There was a greater lipid accumulation in HepG2 cells cultured in human plasma ($F=(1, 8.16)$ 13.71, $P<0.01$). A significant effect of induction was also measured in both media types ($F=(6, 103)$ 28.90, $P<0.001$) and there was a significant interaction between effects of plasma exposure and chemical induction ($F=(6, 10.5)$ 2.94, $P<0.05$). Nile red staining was decreased in HepG2 cells exposed to DBA and indirubin in both media types. Rifampicin exposure also resulted in a decrease in Nile red staining in cells exposed to 10% FFP. There was increased Nile Red binding following exposure to dexamethasone and this effect was significantly higher in cells exposed to FFP ($P<0.05$).

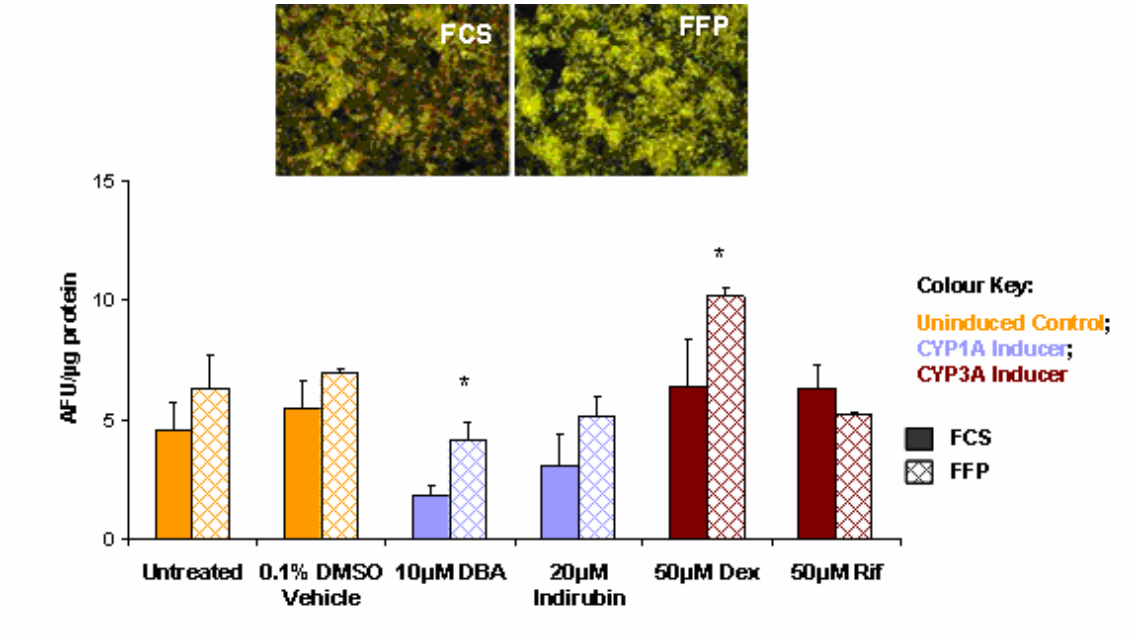


Figure 4-22. Nile red staining of HepG2 cells following culture in αMEM complete containing either 10% FCS or 10%FFP was (top) visualised in uninduced HepG2 cells with a fluorescence microscope and (bottom) quantified in induced HepG2 cells by measuring fluorescence with a cytofluor. Data was analysed by two-way ANOVA with Bonferroni post test. * P< 0.05 when compared to FCS culture (n=3+/-sd).

4.3.3 Discussion

The use of heparin as an anticoagulant within a BAL could affect CYP function and this was investigated in HepG2 cells cultured in 10% FCS. Under basal conditions, heparin alone had no effect on CYP function. In the presence of some inducers, heparin resulted in an increase in ECOD activity but was without effect in other assays. Other authors have reported both positive [235] and negative [236] effects of heparin on CYP function although the former study was performed in rat liver and the latter in porcine hepatocytes.

Once effects of heparin had been determined, the effect of FFP culture on CYP function was considered. ECOD and CYP1A2 activity were comparable following FFP exposure, however, CYP3A activity was decreased in cells cultured in 10% FFP. Beneficial effects of 3D culture were also diminished following FFP exposure.

Isoform specific effects of human plasma exposure on CYP function have been reported in rat hepatocytes, although it should be noted that these cells were cultured in undiluted

plasma and furthermore, species differences should also be taken into consideration. Under basal conditions, plasma improved CYP1A/2B expression and pentoxy- and benzyloxy- resorufin o-dealkylation, but had no effect on ethoxy- or methoxy- resorufin metabolism [237]. The same authors also reported differing effects of FFP on 3MC (reduced induction) and phenobarbital (improved induction) mediated induction.

In this study, effects of FFP on CYP induction were also variable. When ECOD activity was measured, there were inducer dependent effects of both heparin and FFP. Indirubin induction was improved by the presence of heparin in culture medium both in FCS and FFP cultures. Dexamethasone induction was improved by the presence of heparin and then further improved by FFP exposure. DBA and rifampicin mediated induction were both greater in cells cultured in 10% FFP.

CYP1A2 (Luciferin ME) activities were comparable for all media types, however, CYP3A (Luciferin BE) activity was decreased in FFP culture even following induction. This was particularly apparent for AhR agonists indirubin and DBA which suggests that in contrast to the results obtained following FCS culture (section 4.1.2.3), these inducers do not improve CYP3A function in the presence of FFP. There was a slight improvement in CYP3A function in FFP cultures following rifampicin treatment, therefore the effect of this inducer on CYP3A function was maintained in the presence of FFP.

It has previously been reported that, in rat hepatocytes, plasma exposure results in cytoplasmic accumulation of lipid droplets which may have an effect on hepatocyte-specific functions, including urea synthesis [238]; although it was also shown that the negative effects of plasma exposure on function may be improved by media supplementation [239, 240]. Here it was confirmed that human plasma exposure resulted in increased lipid accumulation in HepG2 cells. This was firstly observed when FFP exposed cells were homogenised during the HLM isolation process and was then confirmed by Nile red staining. Effects of lipid accumulation on CYP function has been demonstrated in humans [64, 65, 241]; in PHH prepared from fatty liver (> 40% steatotic) and in cultured PHH that had been treated with free fatty acids to become fat overloaded. In both instances there was a general down-regulation in CYP expression and function (ECOD activity and testosterone metabolism) in steatotic samples. The effect of human plasma on lipid accumulation in HepG2 cells could be contributing to

the decreased CYP3A function that was measured, in this Chapter, following FFP culture, although other components of FFP cannot be discounted. For example, type 1 and type 2 cytokines have both been shown to down-regulate expression of human CYPs and nuclear receptors relevant to drug metabolism [67, 242-244]. Also, many CYP inhibitors act at CYP3A4, and although plasma is pooled from multiple donors to prevent interindividual variation, potentially there could be a xenobiotic presence in plasma which, if not fully removed prior to functional assays, could inhibit enzyme function.

Lipid accumulation following exposure to CYP inducers was also considered. The effect of steroid exposure on hepatic lipid accumulation has been measured by others using Nile red binding assays [234] and was observed here for dexamethasone, particularly when HepG2 cells were cultured in 10% FFP.

Dexamethasone induction has a surprising effect in 3D culture: in both media types, dexamethasone treatment resulted in a decrease in ECOD activity, relative to both vehicle treated cells and monolayer culture. The potential influence of media components on dexamethasone mediated induction will be discussed further in Chapter 6.

Non-specific binding effects of inducers to FFP components could also contribute to effects measured here: the potency of inducers could decrease since there would be less available to bind to nuclear receptors due to this phenomenon. Log P (partition coefficient) is a useful measure of hydrophobicity of compounds and values for the inducers used in this study are summarised below (Table 4-1).

Table 4-1. Calculated Log P values for inducers used in this study. (http://www.syrres.com/esc/est_kowdemo.htm)

Inducer	LogP
DBA	6.7
Indirubin	2.34
Dexamethasone	1.72
Rifampicin	4.24

Lipophilic compounds (those with a higher Log P) have been shown to non-specifically bind to protein [245-247]. This does not appear to have had an effect on induction, since

DBA and rifampicin are both relatively lipophilic but their effect was maintained in FFP culture. Lipid accumulation could be affected by inducers: intracellular accumulation of lipophilic inducers could occur, which could affect induction, or lipophilic compounds could bind fatty plasma extracellularly, which could result in a build up of inducer-lipid complex in the culture media. Possibly lipid accumulation in FFP cultures was reduced in the presence of rifampicin. Alternatively, intracellular lipid accumulation could decrease if inducer binding occurs extracellularly and draws additional lipid from the cells – in both culture media DBA reduced intracellular lipid levels (evidenced by decreased Nile red staining following DBA exposure).

4.4 Conclusion

The results presented in this chapter have demonstrated that HepG2 cells cultured under LG BAL conditions demonstrate Cytochrome P450 function; moreover CYP activity approaching levels measured in primary human hepatocytes can be obtained by manipulating cell culture conditions for HepG2 cells. However, there were certain limitations.

In monolayer culture, chemical induction can improve CYP function. Under basal conditions, ECOD and Luciferin BE metabolism were both markedly improved by 3D culture, however effects of inducers were reduced, which implies that mediators of CYP expression, in particular for CYP3A, are suppressed in HepG2 cells, therefore so is the extent to which CYP activity can be improved.

Although Luciferin BE metabolism in HepG2 cells indicates that they have CYP3A activity, HepG2 cells, even in 3D culture, although able to metabolise testosterone, did not produce the CYP3A4 metabolite 6β-OHTST. This demonstrates that this isoform, which is predominant in PHH, is lacking in HepG2 cells

The improved per bead performance that was achieved following microgravity culture also had a positive effect on HepG2 CYP function, but Luciferin BE metabolism was reduced in HepG2 cells following FBB culture. This can, in part, be attributed to the presence of human FFP in the culture medium. Overall, exposure to FFP had a positive effect on CYP1A induction but resulted in reduced CYP3A activity.

HepG2 cells can be manipulated to perform CYP functions and would readily provide CYP1A function within a BAL, however their CYP3A function is insufficient. HepG2 cell testosterone metabolism is not CYP3A4 mediated and the limited CYP3A function these cells do have is reduced by plasma exposure. In the next Chapter, alternative cell lines will be explored which could be used to supplement CYP3A function within a BAL.

CHAPTER 5 Alternative Cell Lines to Provide Cytochrome P450 Function Within a BAL

As discussed in the previous chapter, the current cellular component of the LG BAL, HepG2 cells, are unable to provide ample CYP3A function; one option would be to introduce different cell types to enhance the function of HepG2 cells.
In primary hepatocytes, it has been demonstrated that co-culture of other cell types, such as stellate cells or fibroblasts, will enhance existing CYP activity [110, 184]. However, since existing CYP function in HepG2 cells is marginal, this approach alone may be insufficient. An alternative would be to use HepG2 cells to provide the synthetic component of the BAL, and an additional cell line to provide CYP activity. In addition to exhibiting superior xenobiotic metabolism capacity (relative to HepG2 cells), in order to be considered fit for purpose, such cells would need to fulfil certain other criteria. Like HepG2 cells they must be: easily expandable, cryopreservable and readily available; maintain their phenotype over multiple passages; and fulfil regulatory requirements (for example come from a demonstrable source). Importantly for this project, any cell line considered must be adaptable to the culture conditions required of the BAL currently under development within the Liver Group.

The aim of Chapter 5, therefore, is to consider alternative cell lines that could potentially be co-cultured with HepG2 cells to provide the detoxificatory pathways required of a BAL. Initially, CYP function in monolayer cells was measured. Further to this, cells were adapted to BAL specific media and finally encapsulation conditions and survival in alginate were profiled.

Two approaches were considered:

i) adapting the human intestinal cell lines Caco2 and LS147T to a 3D culture system and profiling their CYP function under these conditions; and
ii) adapting the HC-04 human liver cell line for use within a BAL and profiling its CYP function under these conditions.

5.1 Cytochrome P450 Activity in Intestinal Cell Lines

In the small intestine, epithelial cells provide the first site of metabolism of orally administered drugs. In humans, there is now a well-recognised role of the small intestine in CYP mediated metabolism and it is widely believed that the intestinal form of CYP3A4 is structurally and functionally identical to the hepatic form [248]. It was therefore hypothesised that human intestinal cell lines which expressed CYP3A4 could potentially provide xenobiotic metabolism in a BAL.

The enterocyte-like cell line Caco2 is best known as a well established *in vitro* model system used to predict oral adsorption of xenobiotics. These cells express CYP3A4, 3A5 and 3A7 mRNA, but under standard culture conditions do not express functional levels of CYP3A [249, 250]. Although it is widely reported that these cells have negligible expression of PXR and show minimal response to PXR ligands, myriad studies have demonstrated that CYP3A4 expression can be induced by 1α,25-dihydroxyvitamin D_3(VD3) [93, 186, 188, 251], which acts through the vitamin D receptor (VDR). Moreover, synergistic effects of Caco2 cells and HepG2 cells in CYP1A mediated EROD metabolism have been demonstrated in a double-layered co-culture system [252] which indicates that these two cell lines would lend themselves favourably to a dual culture approach.

As an alternative intestinal cell line, LS147T cells were also considered. These cells are a trypsinised variant of the LS180 cell line, are goblet-like in structure and as a result produce significant amounts of secretory mucin [253]. As models of drug metabolism both LS180 and LS147T express CYP3A4 and this enzyme can be induced with both PXR and VDR agonists [254-256].

The enterocytic characteristics of Caco2 cells results in a different morphology to the goblet-like secretory LS147T cells (Figure 5-1) and their growth characteristics are quite different.

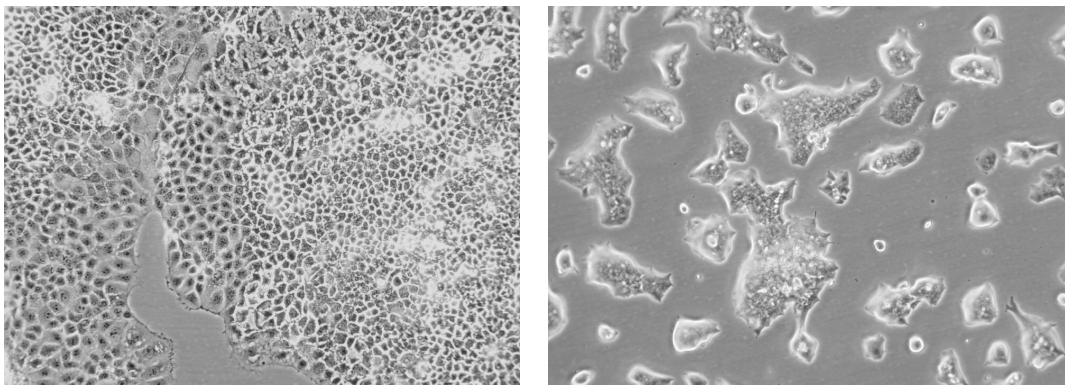


Figure 5-1. Monolayer appearance (x10 magnification) of (left) Caco 2 cells and (right) LS147T cells.

A summary of the two cell types is presented in Table 5-1, and although they differ in many ways, for this thesis, an important shared feature of these two cell lines is their CYP3A4 expression.

Table 5-1. A comparison of intestinal cell lines Caco 2 and LS147T.

Cell Line	Origin	Growth/ Morphology	CYP3A4 Inducer/ Nuclear Receptor	References
Caco2	Colon adenocarcinoma 72 year old Caucasian male	Form confluent differentiated monolayers/ Enterocyte like	VD3/ VDR	188, 253
LS147T	Colon adenocarcinoma 58 year old Caucasian female	Grow in islands Pile on top of one another/ Goblet like	Rifampicin/ PXR VD3/ VDR	253, 255

In this section, monolayer Caco2 and LS147T cells, cultured in supplemented αMEM medium, were used to investigate CYP induction. To determine whether either cell line is suitable for use within a BAL, their growth, viability and CYP3A function was then measured following alginate encapsulation and 3D culture.

5.1.1 Methods

Initially, cells were maintained in DMEM as described in Chapter 2. After cells had been passaged at least once post thawing, and reached 50% confluency, growth medium was switched to complete culture medium as described for HepG2 culture (Chapter 2). Cells were maintained in this medium for at least 3 passages prior to any further studies.

5.1.1.1 Gene Expression

RNA samples were isolated from each cell type following monolayer culture in αMEM complete. Gene expression was performed by qRT-PCR as described in Chapter 2.

5.1.1.2 Monolayer Induction of CYP Function

CYP induction was carried out in Caco2 and LS147T cells in monolayer culture to measure CYP function in these intestinal cell lines. Cells were seeded in 96 well plates at a density of $1x10^4$ cells/well in a volume of 100µl of complete culture medium. Following overnight attachment, cells were treated with CYP inducers for 48 hours and then CYP activity measured by ECOD assay, Luciferin BE assay or Luciferin ME assay (+10µM sulfaphenazole). Cell protein was determined by the BCA assay.

5.1.1.3 Treatment of Caco2 Monolayers with VD3

To assess longer term effects of VD3 exposure Caco2 cells were seeded in 12 well plates at a density of $1x10^4$ cells/well and cultured for 7 days in varying concentrations of VD3 (0.1-2 µM) or 0.1% DMSO vehicle control. CYP activity was measured by Luciferin BE assay and cell protein determined by the BCA assay.

5.1.1.4 Encapsulation of Intestinal Cell Lines

Cells were encapsulated using the Inotech method described in Chapter 2.

5.1.1.4.1 Determination of Encapsulation Conditions

An initial experiment was carried out to determine optimum cell number for encapsulation using Caco2 cells. Cells were resuspended in a 1:1 ratio of HG complete culture medium and alginate at the following densities: 0.5 $x10^6$ cells/ml; 1 $x10^6$ cells/ml; and 2 $x10^6$ cells/ml. 0.25ml/well of beads were added to 6 well plates in a medium volume of 8mls/well. Cells were maintained in static culture for 12 days with medium replenishment every 48 hours. When seeded at 0.5 $x10^6$ cells/ml, proliferation

was poor and very few spheroids formed within beads. When seeded at either 1 x10^6 cells/ml or 2 x10^6 cells/ml, cells proliferated well and viability was maintained, however, at the higher seeding density a large number of cells broke free from the beads (determined by appearance of monolayer cell growth on the bottom of the culture plates). Therefore, 1x10^6 cells/ml was selected as the optimum cell number for further experiments.

5.1.1.4.2 3D Culture of Intestinal Cell Lines

Caco2 or LS147T cells were encapsulated at a density of 1x10^6 cells/ml. Beads were then resuspended in HG medium and added to 6 well plates as above. Cells were maintained in static culture for 8 days with medium changes every other day.

5.1.1.5 CYP Induction in 3D Culture

Following 6 days of 3D culture, cells were treated with 50µM rifampicin for 48 hours. Alternatively cells were treated for 7 days with 1µM VD3. CYP function was measured at day 8 post-encapsulation by Luciferin BE assay, or testosterone metabolism was measured over an 8 hour period.

5.1.2 Results

5.1.2.1 CYP Gene Expression in Caco2 and LS147T Cells

Expression of CYP1A2, CYP3A4 and CYP3A7 were compared in Caco2 and LS147T cells (Figure 5-2). CYP3A4 and CYP1A2 expression were comparable between the two cell lines. CYP3A7 expression was higher (26 fold) in LS147T cells.

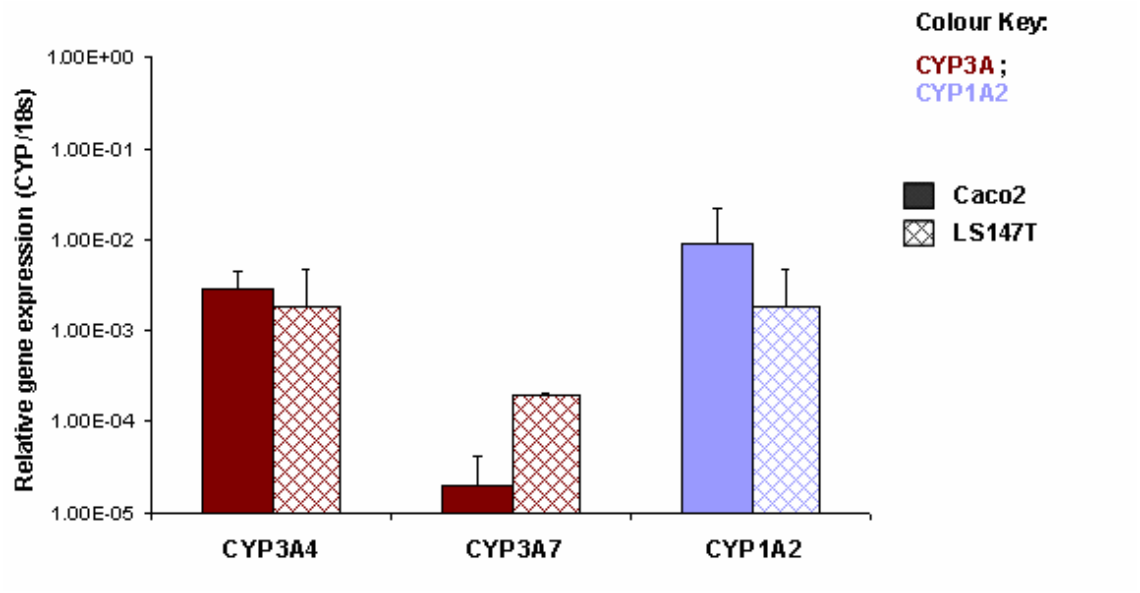


Figure 5-2. CYP expression was measured in mRNA isolated from Caco2 and LS147T cells following monolayer culture. CYP expression is normalised to ribosomal 18s expression and presented as n=2+/- range.

5.1.2.2 Nuclear Receptor Gene Expression in Caco2 and LS147T Cells

There was slightly higher expression of PXR and GR in LS147T cells and higher expression of CAR, RXR and GR in Caco2 cells (Figure 5-3).

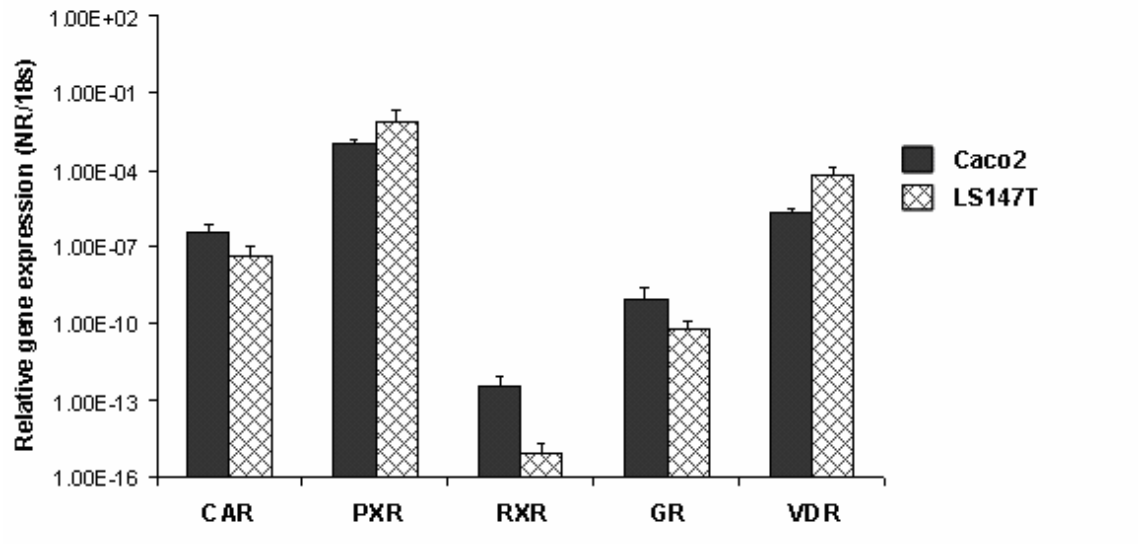


Figure 5-3. Nuclear receptor expression was measured in mRNA isolated from Caco2 and LS147T cells following monolayer culture. CYP expression is normalised to ribosomal 18s expression and presented as n=2+/- range.

5.1.2.3 ECOD Activity in Caco2 and LS147T Cells

ECOD activities were comparable between the two cell lines but were not significantly enhanced by any inducer at the concentrations measured (Figure 5-4).

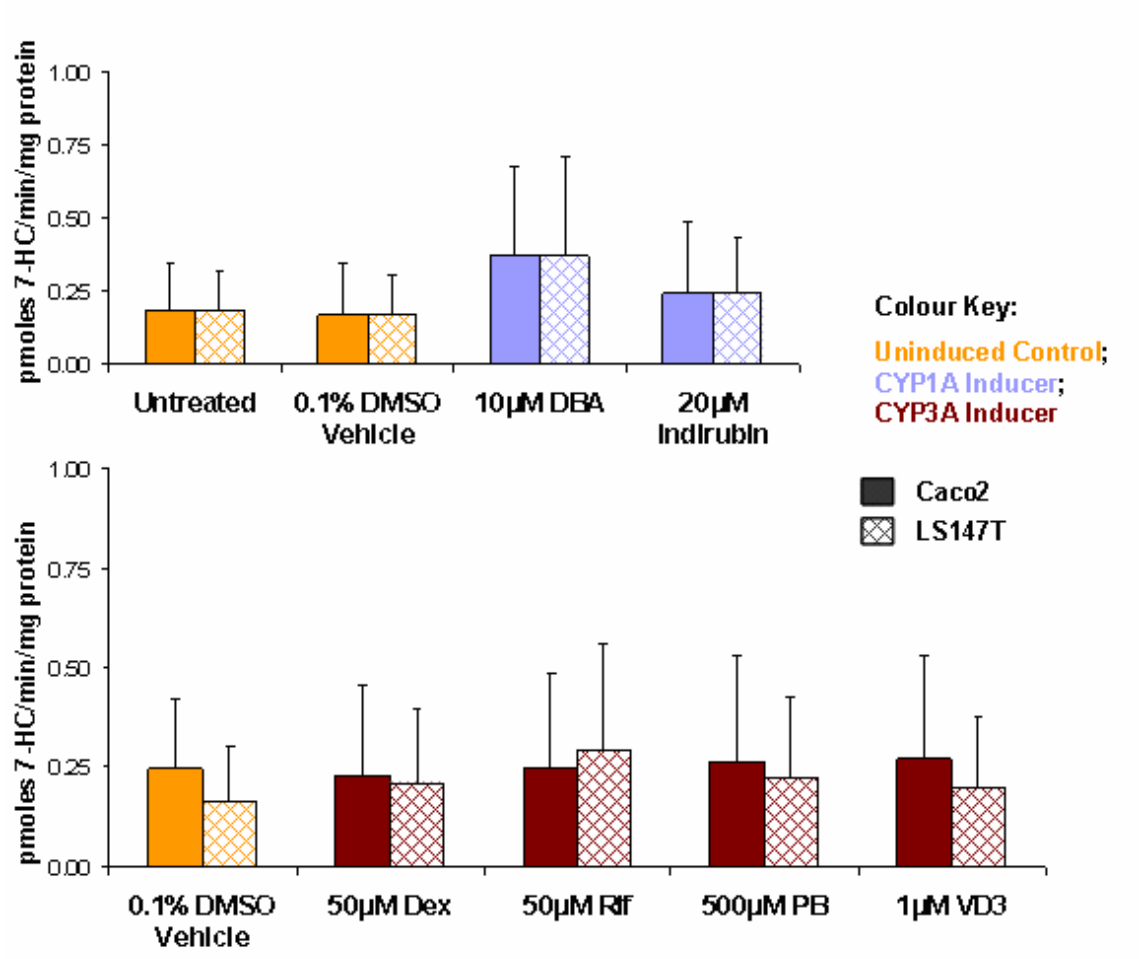


Figure 5-4. ECOD activity was measured in monolayer Caco2 and LS147T cells following 48 hours treatment with (top) CYP1A and (bottom) CYP3A inducers (n=6+/-sd).

5.1.2.4 CYP1A2 Induction in Monolayer Caco2 and LS147T Cells

In monolayer culture, CYP1A2 function was equivalent in Caco2 and LS147T cells (Figure 5-5). In Caco2 cells, there was no significant difference in function following 48 hour incubation with inducers, although 20µM indirubin treatment resulted in a small (1.6 fold) increase in Luciferin ME metabolism above basal. In LS147T cells there was an increase in Luciferin ME metabolism following 10µM DBA treatment (4.0 fold above vehicle); indirubin induction resulted in a significant induction in function in these cells (5.6 fold $P < 0.05$).

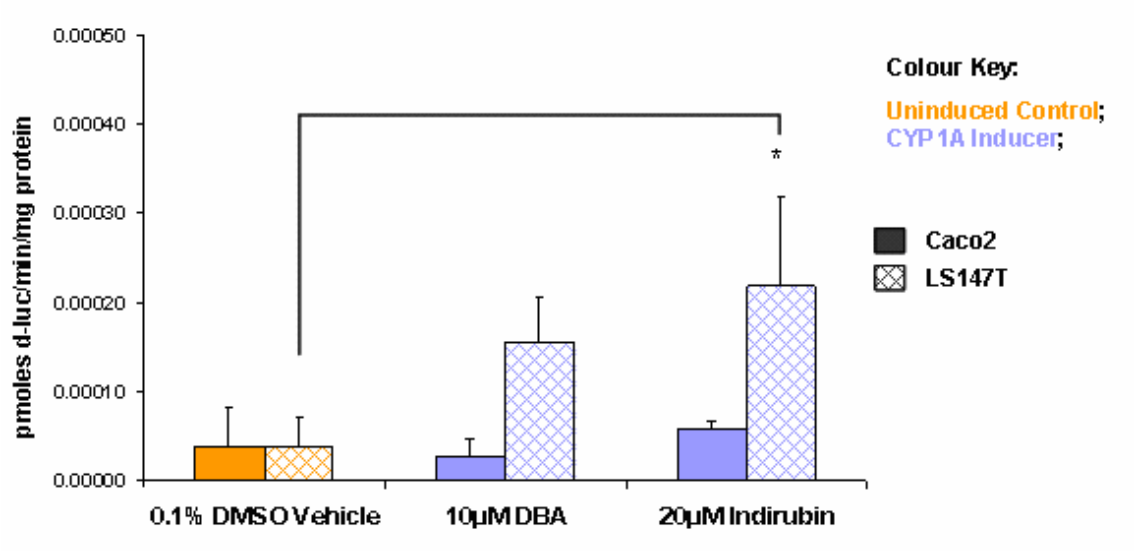


Figure 5-5. Luciferin ME metabolism was measured in the presence of 10µM sulfaphenazole in monolayer Caco2 and LS147T cells following 48 hours induction with 10µM DBA or 20µM indirubin (n=6+/-sd). Data analysis was by one-way ANOVA * P < 0.05 when compared with vehicle treated cells.

Dexamethasone, rifampicin, phenobarbital and VD3 did not affect CYP1A2 function in either cell line.

5.1.2.5 CYP3A Induction in Monolayer Caco2 and LS147T Cells

In monolayer culture LS147T CYP3A (Luciferin BE) activity was not statistically different to the HepG2 CYP3A function that was described in Chapter 4.

When intestinal cell lines were compared, Luciferin BE metabolism was overall significantly higher in LS147T cells than in Caco2 cells independent of the effect of inducers ($P < 0.0001$, $F(1, 0.003) = 92.36$).

In monolayer culture, for both cell lines, the effects of inducers on CYP3A (Luciferin BE) function were not statistically significant (Figure 5-6); although in LS147T cells increased CYP3A function was observed following treatment with dexamethasone (1.9 fold above vehicle), rifampicin (2.8 fold), phenobarbital (2.4 fold) and VD3 (3.1 fold). Caco2 function did not increase with any of these inducers.

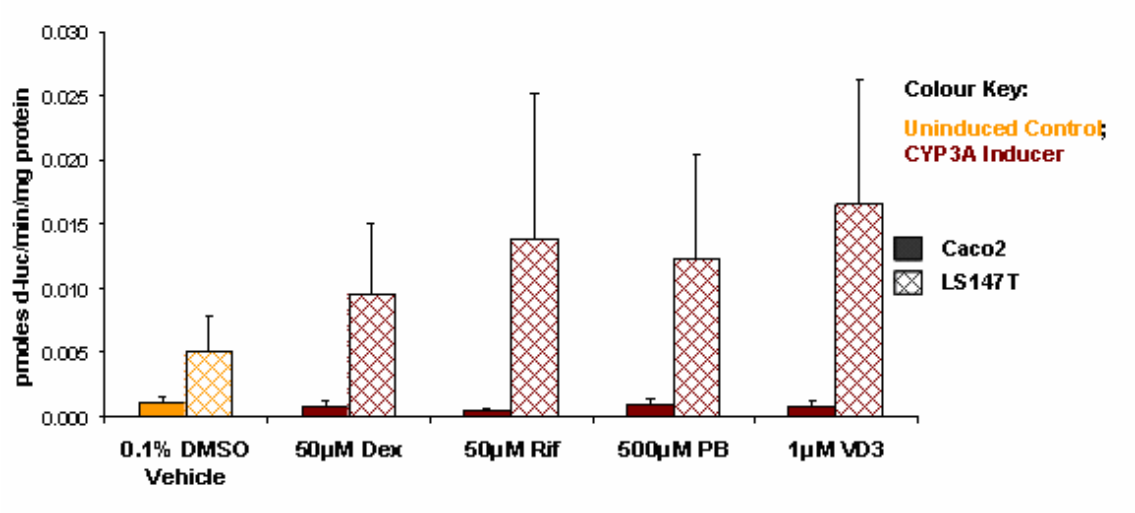


Figure 5-6. Luciferin BE metabolism was measured in Caco2 and LS147T cells following 48 hour treatment with CYP3A inducers (n=6+/-sd).

Since data was normalised during analysis to account for total protein content, the higher level of variability shown in LS147T data can probably be attributed to the manner in which these cells grow on top of each other in monolayer culture and the debris that they produce.

Increased CYP3A activity was also measured in LS147T cells (2.8 +/- 1.1 fold above vehicle) following 10µM DBA treatment and in both Caco2 (1.4 +/- 0.4 fold) and LS147T (3.4 +/- 1.6 fold) cells following 20µM indirubin treatment, although these were not statistically significant changes (Figure 5-7).

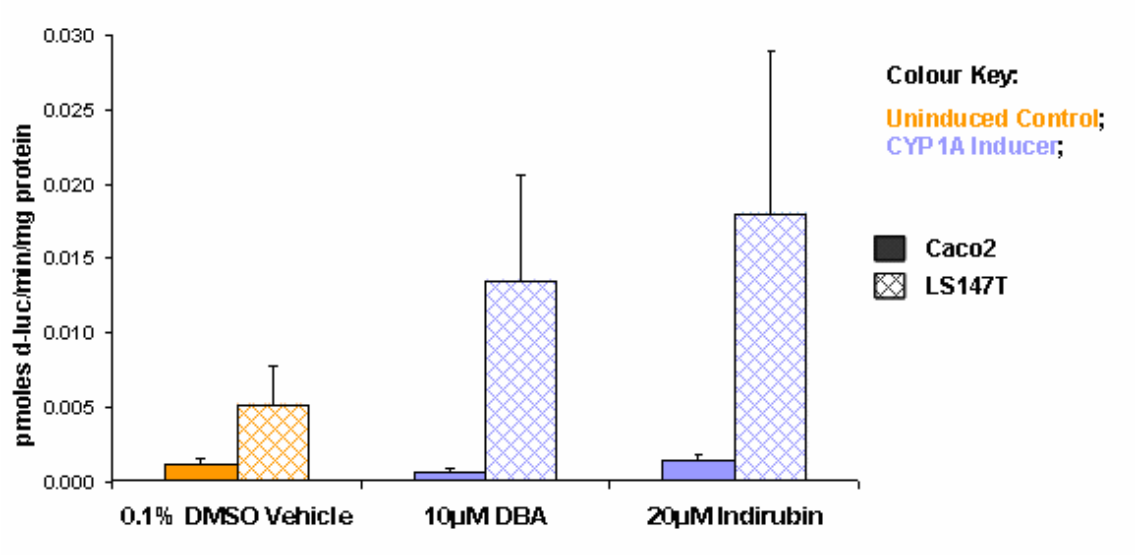


Figure 5-7. CYP3A activity was also improved in Caco2 and LS147T cells following 48 hour treatment with CYP1A inducers (n=6+/-sd).

5.1.2.6 Effect of Prolonged Exposure to VD3 in Caco2 Cells

Others have reported a dose dependent increase in CYP3A expression and function following long-term exposure to VD3 [250]. Luciferin BE metabolism was therefore measured in Caco2 cells that had been exposed to VD3 or 0.1% DMSO vehicle for 7 days (Figure 5-8). Activity in vehicle controls was comparable in Caco2 cells cultured for 7 days or 48 hours (0.0010+/-0.0006 and 0.0011 +/- 0.0005 pmoles d-luc/min/mg protein). Prolonged exposure (7 days) to higher concentrations of VD3 resulted in increased CYP3A activity above DMSO vehicle in Caco2 cells treated with 1µM or 2µM; results were comparable for both concentrations of inducer.

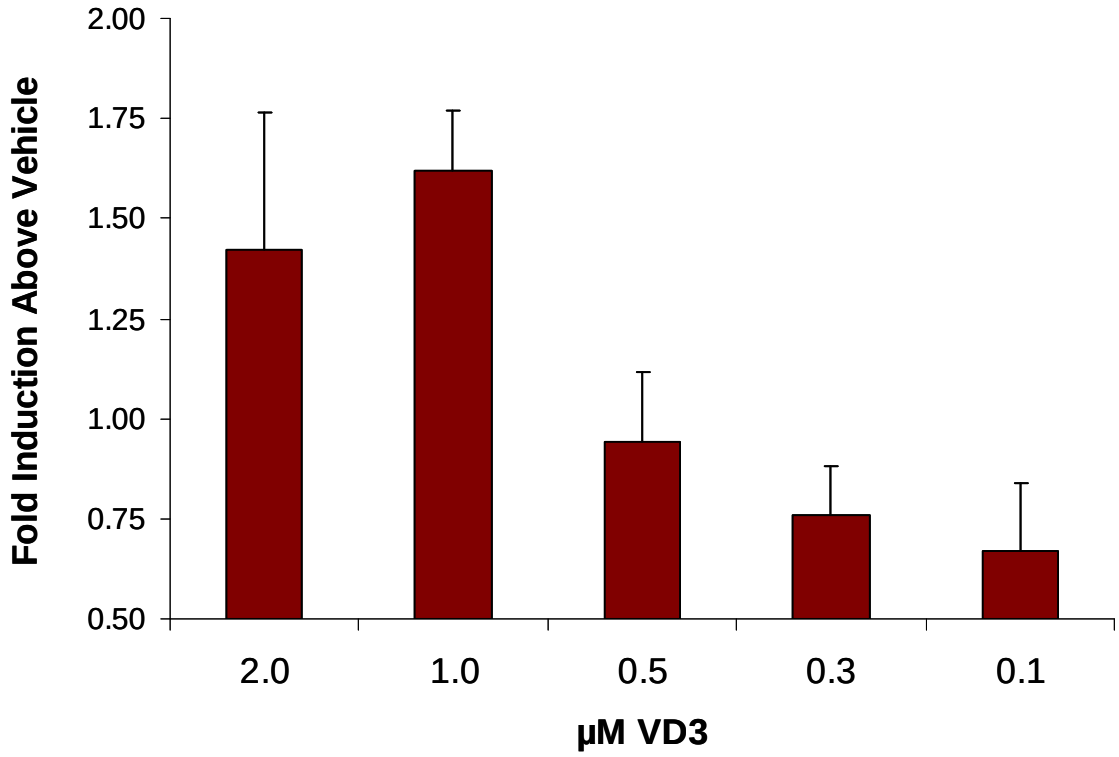


Figure 5-8. An increase in CYP3A activity above 0.1% DMSO vehicle was measured for Caco2 monolayers cultured in a range of VD3 concentrations for 7 days (n=2 +/- range).

5.1.2.7 Alginate Encapsulation of Caco2 Cells

Caco2 cells were maintained for up to 12 days in alginate culture (Figure 5-9). During this time, proliferation remained steady and cell viability was high, as indicated by a minimal level of PI staining.

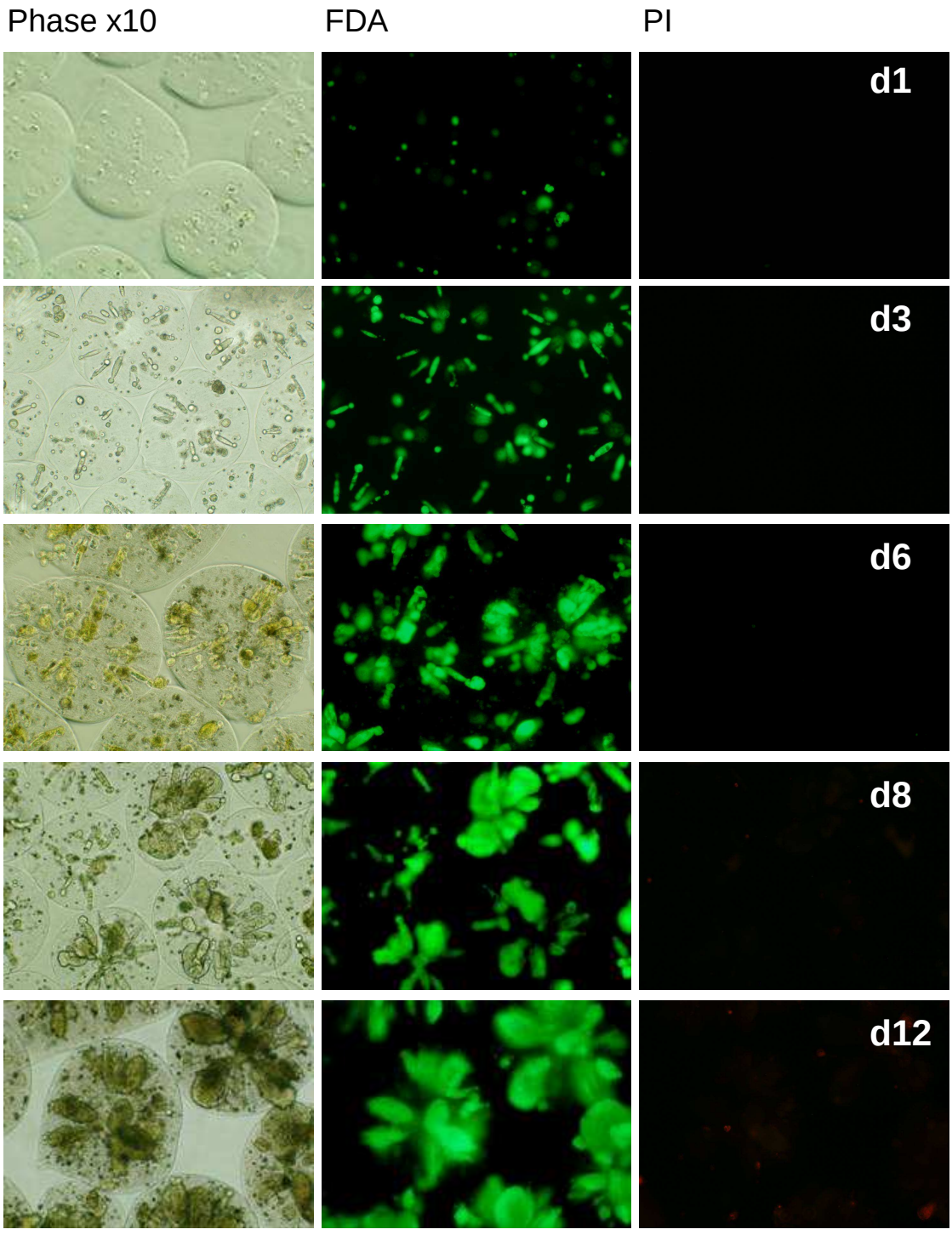


Figure 5-9. Cell viability staining (x10 magnification) of Caco2 cells maintained in 3D static culture for up to 12 days.

5.1.2.8 Alginate Encapsulation of LS147T Cells

LS147T cells were maintained for 8 days in 3D culture. As demonstrated by Figure 5-10, they were able to proliferate. Spheroid formation occurred within 24 hours of encapsulation, however, following encapsulation viability was poor which is indicated by a high level of PI staining and, although there was some recovery in viability during subsequent culture, PI staining remained high throughout.

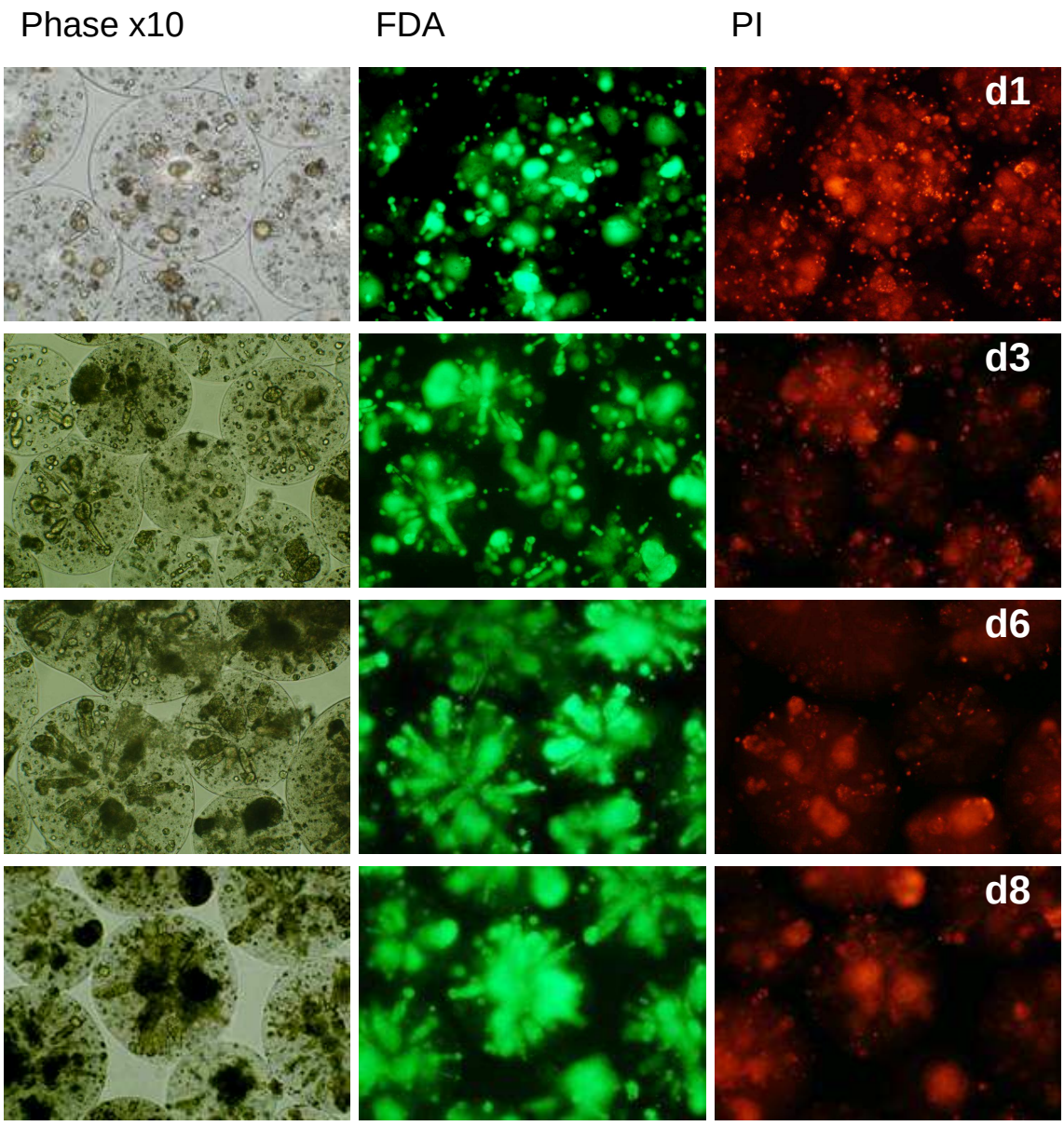


Figure 5-10. Cell viability staining (x10 magnification) of LS147T cells maintained in 3D static culture for up to 8 days.

5.1.2.9 CYP3A Function in 3D Cultures of Caco2 and LS147T Cells

For each cell line, overall there were significant effects of both 3D culture and induction on CYP3A (Luciferin BE) activity. These were cell line dependent and are summarised in Table 5-2.

Table 5-2. Independent and synergistic effects of induction and 3D culture on CYP3A function were examined in Caco2 and LS147T cells. Data was analysed by two-way ANOVA, and significance values P and F (degrees freedom, sum squares) are stated for each variable.

	Caco2	LS147T
Induction	P>0.05 F (2, 2.5^{-5}) = 2.31	**P<0.01** F (2, 0.02) = 6.39
Culture Type	**P<0.001** F (1, 1.5^{-4}) = 27.31	**P<0.05** F (1, 0.01) = 5.43
Interaction Between Variables	**P<0.05** F (2, 4.3^{-5}) = 4.01	**P<0.05** F (2, 0.02) = 4.71

For Caco2 cells, 3D culture alone resulted in a significant increase in Luciferin BE metabolism. Inducer alone has no significant influence on activity, however, there was an interaction between the effects of induction and 3D culture, which resulted in significant improvement in CYP3A activity.

For LS147T cells, significant effects of both 3D culture and induction were observed, moreover, there was also an interaction between the two factors.

5.1.2.9.1 Effects of Induction on CYP3A (Luciferin BE) Activity in 3D Culture

Effects of induction on CYP3A activity in intestinal cell lines are shown in Figure 5-11.

Following rifampicin induction, CYP3A activity was significantly improved in 3D cultures compared to monolayer cultures for Caco2 cells (18 fold $P < 0.001$), although this level of function was 2.1 fold lower than in rifampicin induced 3D cultures of LS147T cells.

Following VD3 induction, CYP3A activity was significantly increased (7 fold, $P<0.01$) in 3D cultures compared to monolayer cultures for LS147T cells.

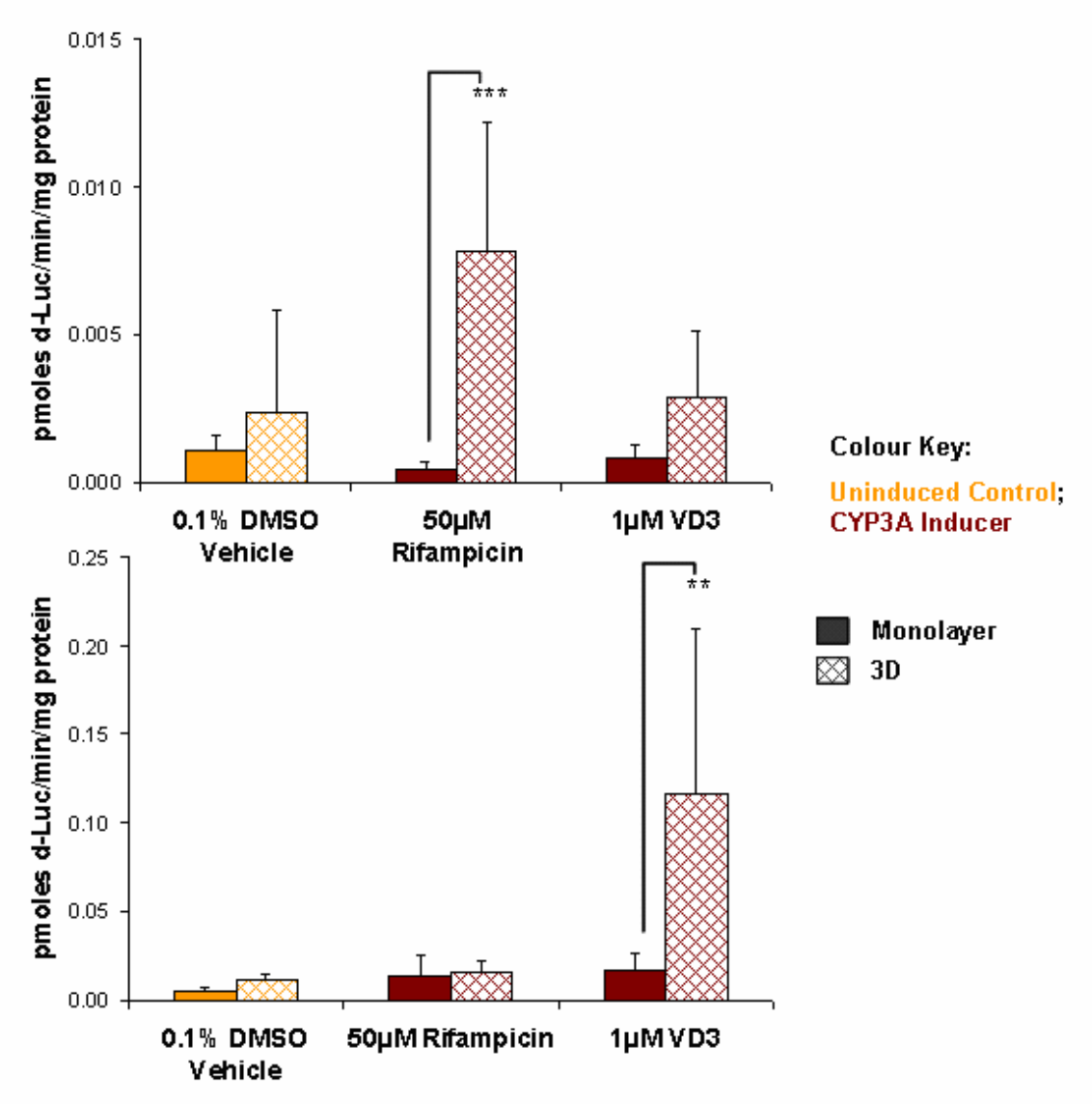


Figure 5-11. CYP3A activity was compared in monolayer and 3D cultures following induction in (top) Caco2 cells and (bottom) LS147T cells (n=6 +/- SD). Note that the scales are different for the top and bottom graphs. Data analysis was by two-way ANOVA with Bonferroni post test. **P < 0.01, *** P <0.001 in 3D cultures compared to monolayer.

5.1.2.9.2 Metabolism of Testosterone by Encapsulated Intestinal Cell Lines

Encapsulated Caco2 or LS147T cells that had been treated, as described above, with either 0.1% DMSO vehicle, 50µM rifampicin or 1µM VD3, were incubated for up to 8 hours with 200µM testosterone. Included in the same analysis run was supernatant from freshly isolated primary human hepatocytes (PHH) incubated for 1 hour with 200µM testosterone.

Caco2 cells did not produce any detectable metabolites of testosterone even after an 8 hour incubation period with the substrate (Figure 5-12).

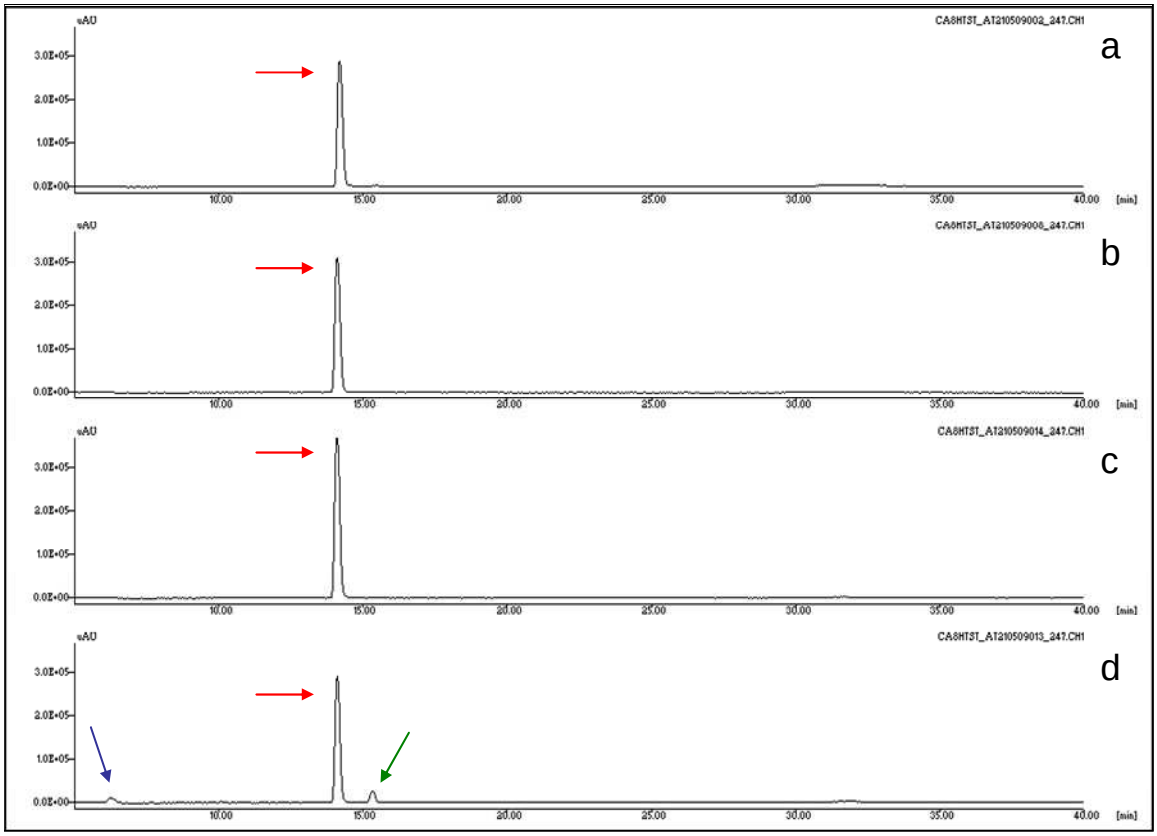


Figure 5-12. HPLC was used to measure metabolism of testosterone in encapsulated Caco2 cells induced with (a) 0.1% DMSO vehicle, (b) 50µM rifampicin or (c) 1µM VD3 and then incubated with 200µM testosterone for 8 hours, or (d) freshly isolated PHH incubated with 200µM TST for 1 hour. TST spike is indicated by →. TST metabolites are indicated by → / → and were measured in PHH only. One representative trace is shown of 5 independent measurements.

After 1 hour incubation with TST, LS147T cells did not produce any detectable metabolite of the substrate. After an 8 hour TST exposure, all LS147T cells had produced a metabolite with the same retention characteristics as one produced by PHH following 1 hour TST exposure. This is indicated in Figure 5-13. Additionally, after 8 hours, LS147T cells treated with 1µM VD3 had also produced a very small amount of 6β-OHTST. However, this peak size lay below the limits of the standard curve that had been defined for the assay and so could not be quantified.

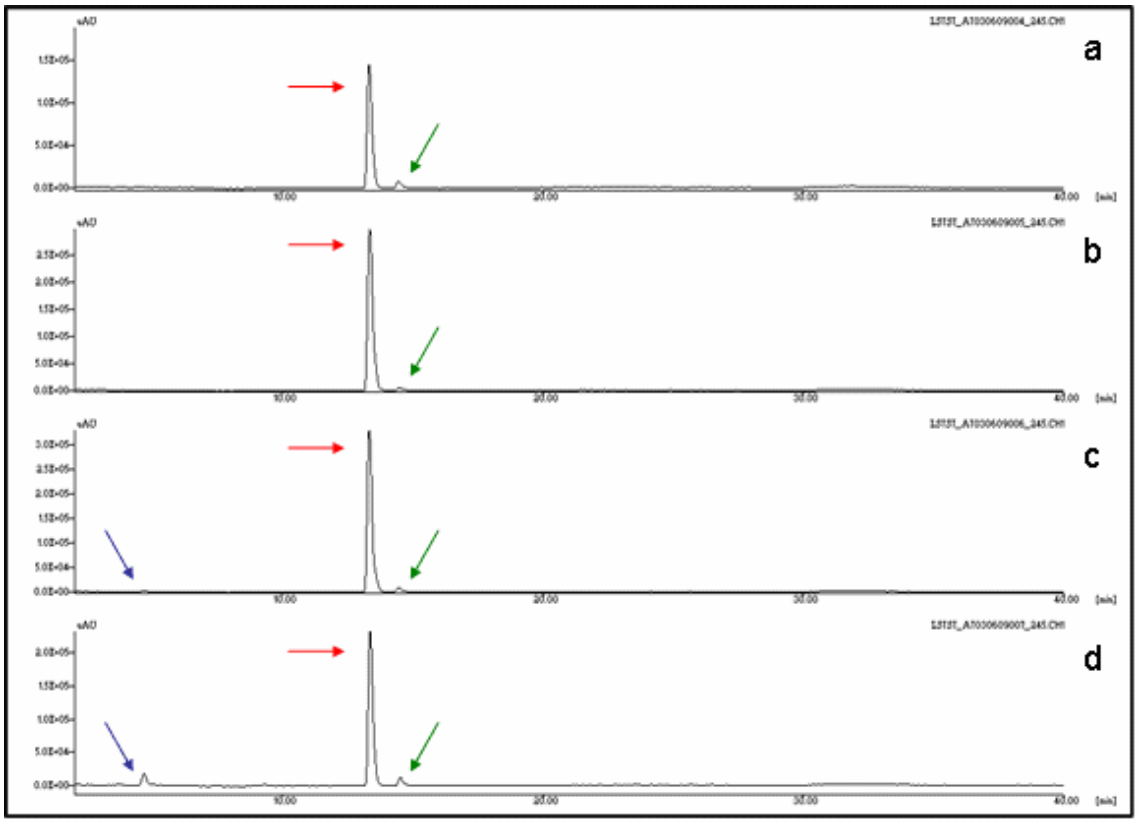


Figure 5-13. HPLC was used to measure metabolism of testosterone in encapsulated LS147T cells induced with (a) 0.1% DMSO vehicle, (b) 50µM rifampicin or (c) 1µM VD3 and then incubated with 200µM Testosterone for 8 hours, or (d) freshly isolated PHH incubated with 200µM testosterone for 1 hour. Testosterone spike is indicated by →. 6β-OHTST metabolite is indicated by → and was measured only in PHH and VD3 cells after 8 hour TST incubation. A further metabolite is indicated by → and was detected in PHH and all LS147T cells only after an 8 hour TST incubation. One representative trace is shown of 5 independent measurements.

5.1.2.10 A Comparison of PHH, HepG2 and Intestinal Cell Lines

CYP3A activity in LS147T and Caco2 cells was compared with activity measured in freshly isolated PHH (results from Chapter 3) and with HepG2 cells treated with rifampicin (results from Chapter 4). LS147T CYP3A function was highest in 3D culture following treatment with 1µM VD3 and was higher than measured in HepG2 cells and the average value measured in PHH. CYP3A activity in Caco2 cells was highest in 3D culture following treatment with 50µM rifampicin but was lower than in HepG2 cells and freshly isolated PHH (Table 5-3).

Table 5-3. CYP3A (Luciferin-BE) activity was higher in freshly isolated primary human hepatocytes than in Caco2 cells but not LS147T cells following 3D culture. Data was compared from PHH isolated from 9 separate donors assayed in quadruplicate and monolayer and 3D cultures of HepG2 (48 hours 50µM rifampicin), Caco2 cells (48 hours 50µM rifampicin) or LS147T cells (48 hours 1µM VD3). Data is expressed as pmoles d-luc/min/mg protein (mean+/-SD for cell lines, mean, median and range for PHH).

Cell Type	**CYP3A Activity (pmoles d-luc/min/mg protein)**	
PHH Average (9 donors)	0.071	
PHH Median (9 donors)	0.051	
PHH Range (9 donors)	0.022 - 0.167	
	Monolayer	**3D**
HepG2 + Rifampcin	0.019 +/-0.002	0.028 +/-0.007
Caco2 + Rifampicin	0.0004 +/-0.0002	0.0078 0.0044
LS147T + VD3	0.017 +/-0.010	0.117 +/- 0.092

5.1.3 Discussion

In this Chapter, expression and function of CYP1A2 and CYP3A were demonstrated in both Caco2 and LS147T cells. In both cell lines, mRNA expression data demonstrates that CYP3A4 expression predominates over CYP3A7 although there was a greater expression of CYP3A7 in LS147T cells than in Caco2 cells. Expression profiles of nuclear receptors (NR) were also examined and there were slight differences between the cell lines: VDR and PXR expression were slightly higher in LS147T cells and CAR, GR and RXR expression were higher in Caco2 cells. Others have reported that LS147T cells are lacking in CAR and Caco2 cells are lacking in PXR expression [251, 255]. These discrepancies could be ascribed to both cell source and the culture media used in these studies, which may affect both CYP and NR expression in intestinal cell lines [55].

In monolayer culture, ECOD activity was comparable for both cell lines but was not improved by induction. CYP1A2 function was also equivalent in uninduced cells, however, a greater induction was observed in LS147T cells following indirubin, and to a lesser extent DBA, exposure. CYP3A function was also superior in monolayer LS147T cells to Caco2 cells; Caco2 CYP3A function was only improved following prolonged (7 day) exposure to VD3. For both Caco2 and LS147T cells, a slight effect of CYP1A2 inducers on Luciferin BE function was also observed. This implies either that these inducers are able to act as ligands at NR which mediate CYP3A function such as PXR or CAR, or NR cross-talk between mediators of CYP1A2 and CYP3A. This will be investigated further for HepG2 cells in Chapter 6.

Variations in Caco2 function that are dependent on differentiation state and passage number have been presented by a number of researchers [248, 250, 257, 258]. Here, it is shown that Caco2 cells responded well to alginate encapsulation and that 3D culture resulted in significant improvement in CYP3A function of this cell line. Importantly, it was demonstrated in 3D culture that CYP3A function was better upregulated by 48 hour induction with 50µM rifampicin than by long-term treatment with 1µM VD3. This contradicts not only research reported by other groups, but also the monolayer results described above.

It has previously been reported that CYP3A4 induction in Caco2 cells can be induced by VD3 and not by rifampicin [188, 258]. It is also proposed that in Caco2 cells, CYP3A4 function is dependent upon ECM, serum and long-term VD3 exposure [250]. One role these authors suggest for VD3 is that it acts as a precursor of cellular differentiation; it may be that other components of LG complete αMEM were also able to perform this role. This, combined with the effects of 3D culture on cellular architecture (resulting in improved cell-cell communication and ECM deposition [259]), could be sufficient to promote rifampicin mediated induction in Caco2 cells.

Culture media dependent CYP3A function of Caco2 cells has previously been reported by Schmiedlin Ren et al [250], although changes in function reported by these authors were moderately low. Caco2 cells profiled in this thesis were initially cultured in DMEM but were adapted to αMEM containing supplements, which may have had an effect on cellular differentiation and CYP expression, including hydrocortisone, insulin, TRH and sodium selenite.

Raeissi [249] demonstrated by RT-PCR that CYP3A5 is the predominant form of CYP in uninduced Caco2 cells maintained in DMEM. One other possibility is that other isoforms of CYP3A (CYP3A5/CYP3A7) are the predominant isoforms induced in 3D culture following rifampicin induction, and that CYP3A4 is not the predominant functional isoform in Caco2 cells under these culture conditions. Although it has been demonstrated that rifampicin has a minimal effect on CYP3A5 (and CYP3A4) expression in Caco2 cells [55], it is plausible that 3D culture has an influence on CYP3A7 expression in Caco2 cells. This could account for the decreased effect of VD3 induction in 3D culture relative to rifampicin, since it has been reported that VD3 has a minimal effect on CYP3A7 (and CYP3A5) induction and is a greater inducer of CYP3A4 [93, 188]. It could also account for why Caco2 cells are unable to produce 6β-OHTST even following 3D culture, CYP induction and prolonged (8 hour) TST exposure.

The hypothesis that rifampicin was inducing CYP3A7 in 3D cultures of Caco2 cells is further strengthened by other reports which suggest that expression of PXR in Caco2 cells is negligible [251], or that PXR ligands do not improve CYP3A4 function in these cells. NR expression data presented in section 5.1.2.2 demonstrated that PXR is expressed in the Caco2 cells used in this study; possibly, rifampicin caused induction of

CYP3A7 by acting at PXR [138]. Alternatively, rifampicin could have acted at GR or CAR both of which could cause induction of CYP3A7.

Effects of 3D culture on LS147T CYP3A function were different to those observed in Caco2 cells. The influence of rifampicin in 3D culture was negligible, whereas encapsulated LS147T exposed to VD3 demonstrated significantly increased Luciferin BE metabolism, and also appeared to produce the CYP3A4 metabolite 6β-OHTST. This both confirms the selectivity of the Luciferin BE substrate for CYP3A and suggests a dominant role of CYP3A4 metabolism in this cell type. A greater effect of VD3 relative to rifampicin on CYP expression has been reported by others in LS180 cells [258], which are a variant of LS147T cells, and it is postulated that CYP3A4 induction in response to VD3 is a characteristic that is typical to intestinal cell lines. Both of these factors are supported by the evidence that VD3 induction is mediated by VDR, which is expressed in higher amounts in the intestine than the liver [251].

Unlike Caco2 cells, viability of LS147T cells appeared poor in 3D culture. This is likely to be a replication of their properties in monolayer culture where they shed a lot of debris. In monolayer culture, this debris can be mechanically cleared from cultures by frequent media changes however, in 3D culture, cell debris appears to be held within the alginate beads for prolonged periods of time. One effect of this was decreased per bead performance. Function of LS147T cells was significantly improved in 3D culture, however data was reported per mg protein. If this value were corrected to account for viable cell number only, functional activity, and hence per bead performance, would be higher than the values reported here. Potentially 3D culture of LS147T cells could be optimised through microgravity culture; improved media flow would increase the nutrient availability and removal of toxic waste products. However, the amount of debris produced by LS147T cells would remain problematic when considering these cells for BAL use.

As described above, differences in CYP function were observed for both cell lines, and many factors could account for this, not least their original site of isolation. Both cell lines are adenocarcinoma derived and secrete carcinoembryonic antigen, which is detected in humans as a marker of tumour formation. As described for HepG2 cells, normal cellular functions may not be conserved by these cell lines. Furthermore, it has been demonstrated that intestinal CYP3A4 function is affected by disease; although

CYP3A expression was not directly affected by presence of tumour in the large intestine [260], testosterone metabolism was decreased in duodenal biopsy samples taken from a paediatric coeliac population, and was then recovered following treatment [261], and this may account for some of the discrepancies measured between the two cell lines since neither should be considered as a model of healthy human intestine [249, 262]. Additionally, cell source and culture media could equally account for some of the properties of these cells.

In this chapter, it has been demonstrated that 3D culture has a beneficial effect on function of intestinal cell lines. Others have reported that when Caco2 cells are cultured on ECM and stimulated to produce differentiated monolayers, under certain conditions, these cells have a tendency to form complex structures rather than maintain a monolayer growth pattern [250]. LS147T cells, even in monolayer culture, grow on top of each other forming their own structures. Both of these factors indicate that these cell lines would favour a 3D culture environment for optimum function.

Caco2 cells arguably adapted better to 3D culture than LS147T cells in terms of maintained viability, however, their function was not comparable to PHH even after induction. Intestinal CYP expression, *in vivo,* is far lower than in the liver and this was reflected in Caco2 cell function. The advantages of marked upregulation of CYP3A4 function in LS147T cells following 3D culture and VD3 exposure were offset by the large amount of cell debris produced by this cell line. Additionally, intestinal cell lines can only offer CYP function to a BAL rather than the range of hepatic functions that could potentially be offered by a cell line of hepatic, origin and so for these reasons a liver derived cell line remains the most appealing option.

5.2 Cytochrome P450 Activity in the HC-04 Liver Cell Line

Hep G2 cells are of tumor origin and, as is often the case with cancer cells, do not retain normal hepatocellular metabolising capacities, most likely as a result of dedifferentiation. Immortalised human hepatocytes from normal liver could offer a solution as, unlike primary cells, they are easily expandable and may maintain a fuller range of hepatocyte functions. The spontaneously immortalised human hepatocyte cell line HC-04 functionally expresses the major CYPs, in addition to nuclear receptors, enzymes involved in Phase II metabolism and hepatic transcription factors [160]. These cells were derived from normal liver following resection of hepatoma. Unlike many immortalised hepatocyte cell lines, HC-04 cells were generated without the use of simian virus 40 large tumor antigen (SV40) transfection or co-culture with liver epithelial cells, both of which are methods that could be problematic for BAL relevant cell lines.

Morphologically, these cells are not dissimilar to HepG2 cells (Figure 5-14). CYP3A4 protein expression can be increased in HC-04 cells by the PXR agonist rifampicin, and these cells are able to metabolise the CYP3A4 substrate midazolam, although their reported drug metabolising capacities were equivalent only to PHH from donors with low levels of CYP3A4 function [160].

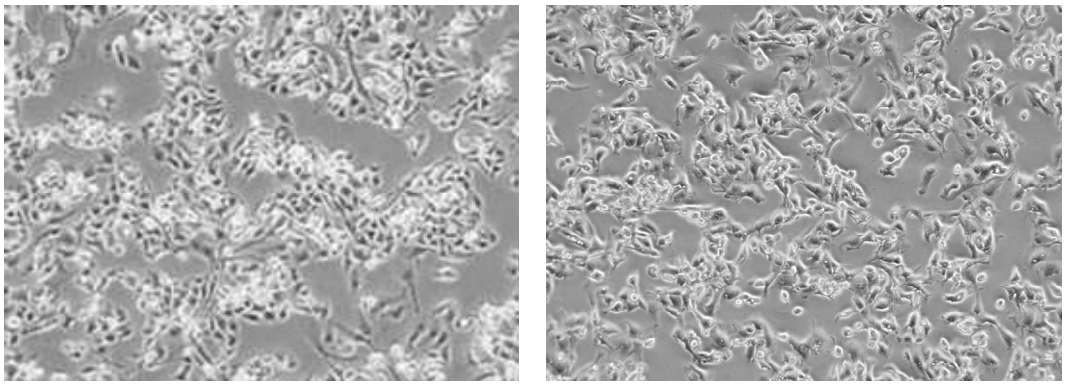


Figure 5-14. Monolayer appearance (x10 magnification) of (left) HC-04 cells compared to (right) HepG2 cells.

The suitability of HC-04 cells for use in a BAL has not so far been evaluated. The conditions under which they were developed would be unsuitable for large scale 3D culture. This cell line was developed on an extracellular matrix (ECM) consisting of

collagen and fibronectin, and the culture medium that they were developed in (HCM, Chapter 2), in addition to being costly, contains supplements, including dexamethasone and growth factors, which may alter CYP activity as discussed in previous chapters. With respect to HC-04 cells therefore, there were several questions to address.

i) Will HC-04 cells attach to tissue culture plastic and proliferate in LG BAL developed αMEM?
ii) Are CYP activities comparable in HC-04 cells cultured +/- ECM in αMEM or HCM?
iii) Is CYP function maintained over multiple passages in BAL relevant culture conditions?
iv) Can HC-04 cells be encapsulated in alginate and maintained in 3D culture?

With regard to points (i) and (ii), it subsequently transpired that the HC-04 cells used to carry out this work were supplied from the original source heavily contaminated with mycoplasma and this affected cell function, with a resultant loss in CYP function. This data is therefore not included in this Chapter but is presented in Appendix A. The following work was carried out on HC-04 cells cultured on tissue culture plastic, adapted to αMEM complete and treated with BM-Cyclin which successfully eliminated mycoplasma contamination. To ensure that no contamination remained, cells were tested weekly for mycoplasma by PCR whilst the following experiments were carried out.

5.2.1 Methods

5.2.1.1 CYP Induction in Monolayer Culture

HC-04 cells ($2.5x10^4$ cells/well in a volume of 100µl) were added to 96 well plates. Following overnight attachment, cells were treated with CYP inducers for 48 hours and then CYP activity measured by either ECOD assay, Luciferin BE assay or Luciferin ME assay (measured in the presence of 10µM sulfaphenazole). Protein content was determined by the BCA method.
This experiment was performed weekly over a period of one month to determine whether activity was maintained during time in culture in adapted culture conditions.

5.2.1.2 Metabolism of Testosterone

HC-04 cells were plated out at a density of $2.5x10^4$ cells/well, allowed to attach overnight and then incubated with either 0.1% DMSO vehicle or 50µM rifampicin for 48 hours. Cells were subsequently incubated at 37ºC with 200µM testosterone for 8 hours; supernatant was removed for analysis after 1 hour and 8 hours. Protein content was determined by the BCA method.

5.2.1.3 Encapsulation of HC-04 Cells

HC-04 cells were revived from liquid nitrogen and cultured in T175 culture flask without collagen matrix in αMEM complete. Once confluent, cells were encapsulated by Inotech encapsulation as described in Chapter 2. Initially cells were encapsulated at $0.5x10^6$ cells /ml, however, at this density cell proliferation and spheroid formation was poor and therefore $1x10^6$cells/ml was selected as the seeding density for further experiments. Beads were resuspended at a ratio of 0.25ml beads: 8ml HG medium and maintained in static culture for 8 or 15 days. Medium was replenished every other day and viability and cell growth assessed by FDA/PI staining.

5.2.1.4 CYP Induction in 3D Culture

At day 6 or day 13 post encapsulation, cells were treated with 0.1% DMSO vehicle or 50µM rifampicin in HG medium. Following a 48 hour induction period, CYP function was measured by Luciferin BE assay.

5.2.1.5 Gene Expression in HC-04 Cells

RNA was isolated from HC-04 cells that had either been maintained in monolayer culture or grown in 3D culture for 8 days and in both instances treated for 48 hours with either 0.1% DMSO vehicle or 50µM rifampicin. Following DNase I treatment and cDNA preparation (Chapter 2), gene expression was determined by qRT-PCR.

5.2.2 Results

Over the month-long period in which their function was measured, HC-04 cells maintained their activity. The following data is therefore presented as the average function from the four weekly experiments, performed in quadruplicate, +/-SD.

5.2.2.1 ECOD Activity in HC-04 Cells

ECOD activity in monolayer HC-04 cells was significantly increased (41 fold P< 0.001) following indirubin mediated induction. ECOD activity was also increased following DBA induction (5.2 fold) however this change was not significantly different to vehicle treated cells (Figure 5-15).

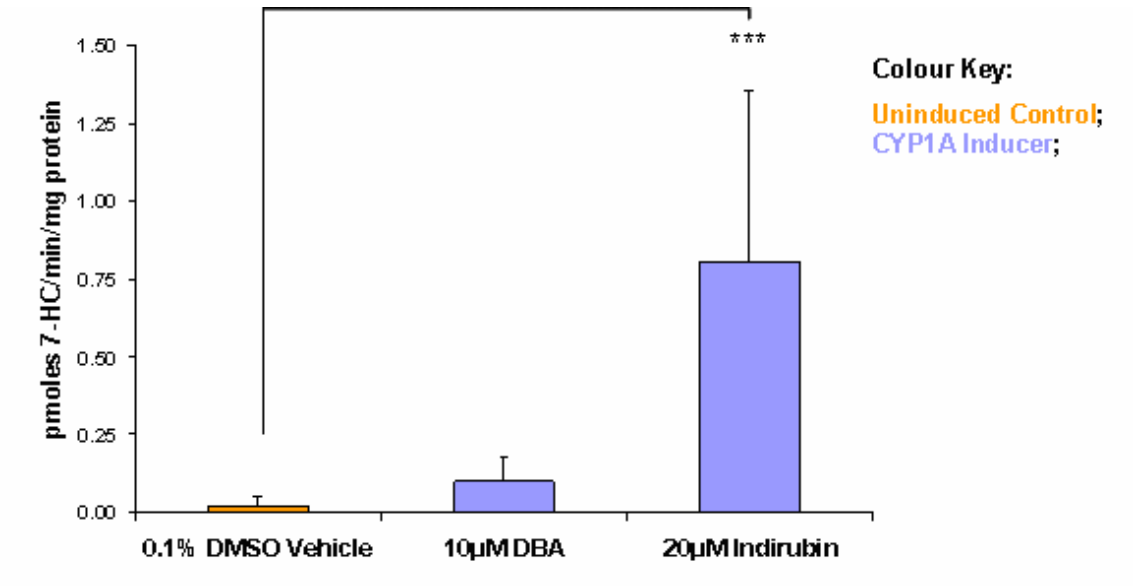


Figure 5-15. ECOD activity was measured in monolayer HC-04 cells following 48 hours induction with 10µM DBA or 20µM indirubin. Data analysis was by one-way ANOVA. *** P < 0.001 when compared to vehicle treated cells.

CYP3A inducers dexamethasone, rifampicin and phenobarbital did not affect ECOD activity in HC-04 cells.

5.2.2.2 CYP1A2 induction in Monolayer Culture

Luciferin ME metabolism in monolayer HC-04 cells was significantly increased (5.2 fold, P< 0.001) following indirubin mediated induction. There was a slight but insignificant increase in CYP1A2 activity following DBA mediated induction (1.5 fold), but this change was not significantly different to the vehicle treated cells (Figure 5-16).

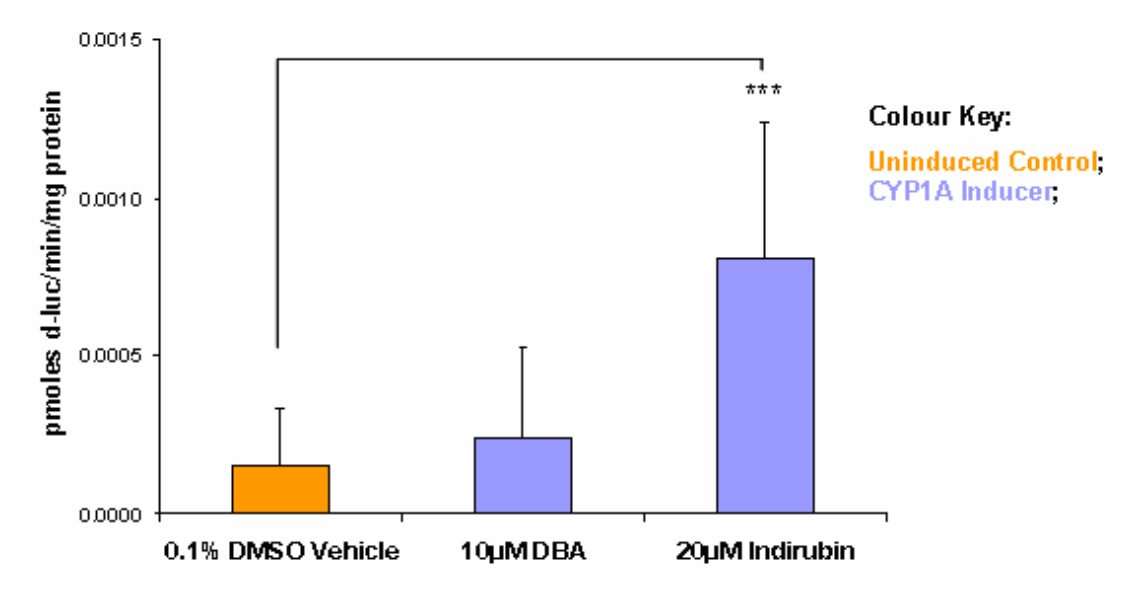


Figure 5-16. Luciferin ME metabolism was measured in the presence of 10µM sulfaphenazole in monolayer HC-04 cells following 48 hours induction with 10µM DBA or 20µM indirubin. Data analysis was by one-way ANOVA. *** P < 0.001 when compared with vehicle treated cells.

CYP3A inducers dexamethasone, rifampicin and phenobarbital did not affect CYP1A2 function in HC-04 cells.

5.2.2.3 CYP3A Function in Monolayer Culture

Effects of CYP3A inducers on CYP3A (Luciferin BE) activity are shown in Figure 5-17. Dexamethasone did not alter CYP3A function in HC-04 cells. Moderate induction was observed following treatment with phenobarbital (1.3 fold above vehicle) and rifampicin (1.4 fold above vehicle), however, neither of these results were statistically significant.

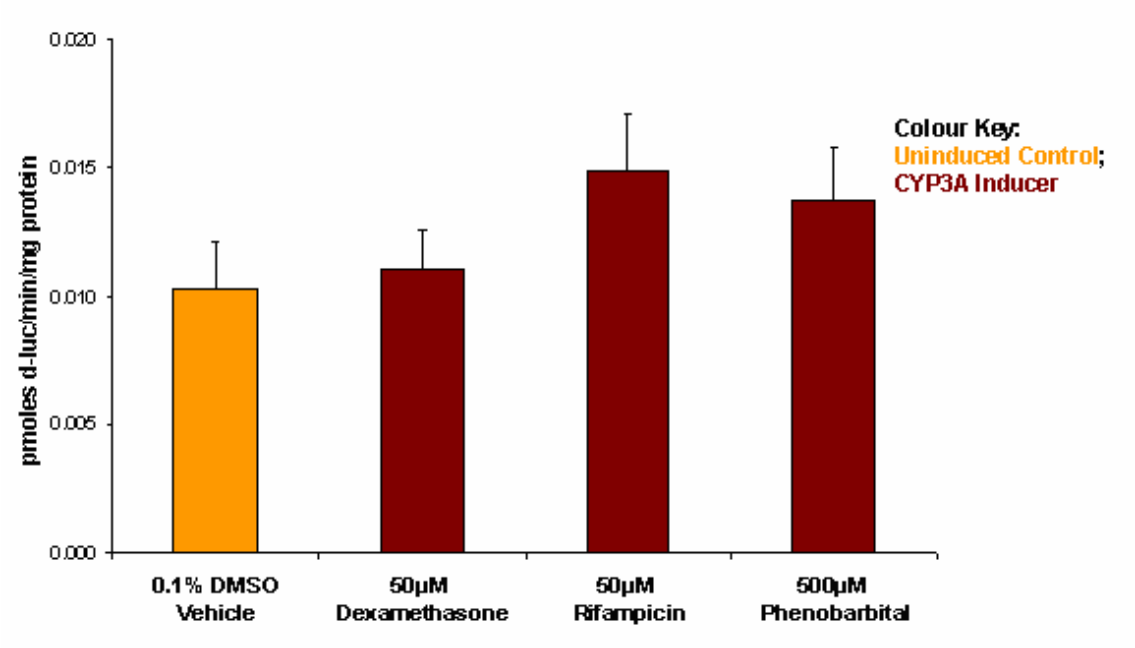


Figure 5-17. CYP3A (Luciferin BE) activity was measured in monolayer HC-04 cells following 48 hour treatment with CYP3A inducers.

CYP1A inducers DBA and indirubin had no effect on CYP3A (Luciferin BE) activity in HC-04 cells.

5.2.2.4 Metabolism of Testosterone

HC-04 cells treated for 48 hours with 0.1% DMSO vehicle or 50µM rifampicin were incubated for up to 8 hours with 200µM testosterone. Included in the same analysis run was supernatant from freshly isolated PHH, incubated for 1 hour with 200µM testosterone. Unlike data obtained with PHH, HPLC analysis of testosterone metabolism by HC-04 cells did not indicate metabolism of TST or detectable amounts of 6β-OHTST after a 1 hour exposure. Following 8 hours of TST exposure, HC-04 cells that had been treated with rifampicin produced a detectable amount of a TST metabolite that was also produced by PHH (Figure 5-18).

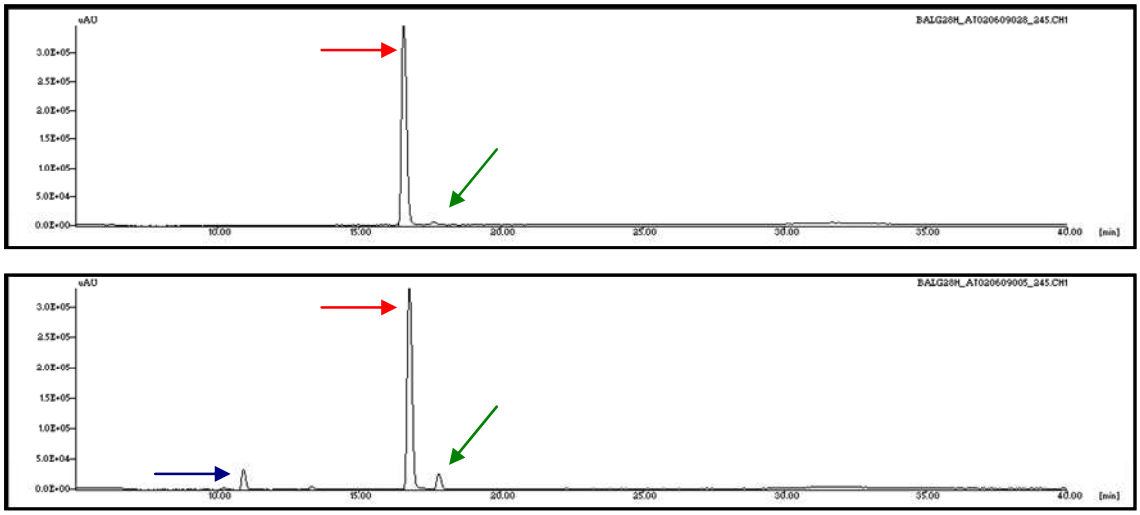


Figure 5-18. Measurement of testosterone metabolism in (top) rifampicin treated HC-04 cells incubated for 8 hours with 200µM testosterone and (bottom) freshly isolated PHH incubated for 1 hour with µM testosterone. Testosterone peaks are indicated by →. Production of 6β-OHTST metabolite is indicated by → and was observed only in PHH. A further metabolite → was detected in PHH and in HC-04 cells only after rifampicin treatment and 8 hour incubation with 200µM TST. One representative trace is shown of 4 independent measurements.

5.2.2.5 3D Culture of HC-04 Cells

Figure 5-19 shows the growth and viability of HC-04 cells when cultured in 3D format for up to 15 days. Proliferation and spheroid formation appeared slower than in HepG2 cells over 8 days of culture but viability was maintained throughout this culture period. Although improved spheroid formation was observed following prolonged (15 days) culture, cell viability was markedly decreased.

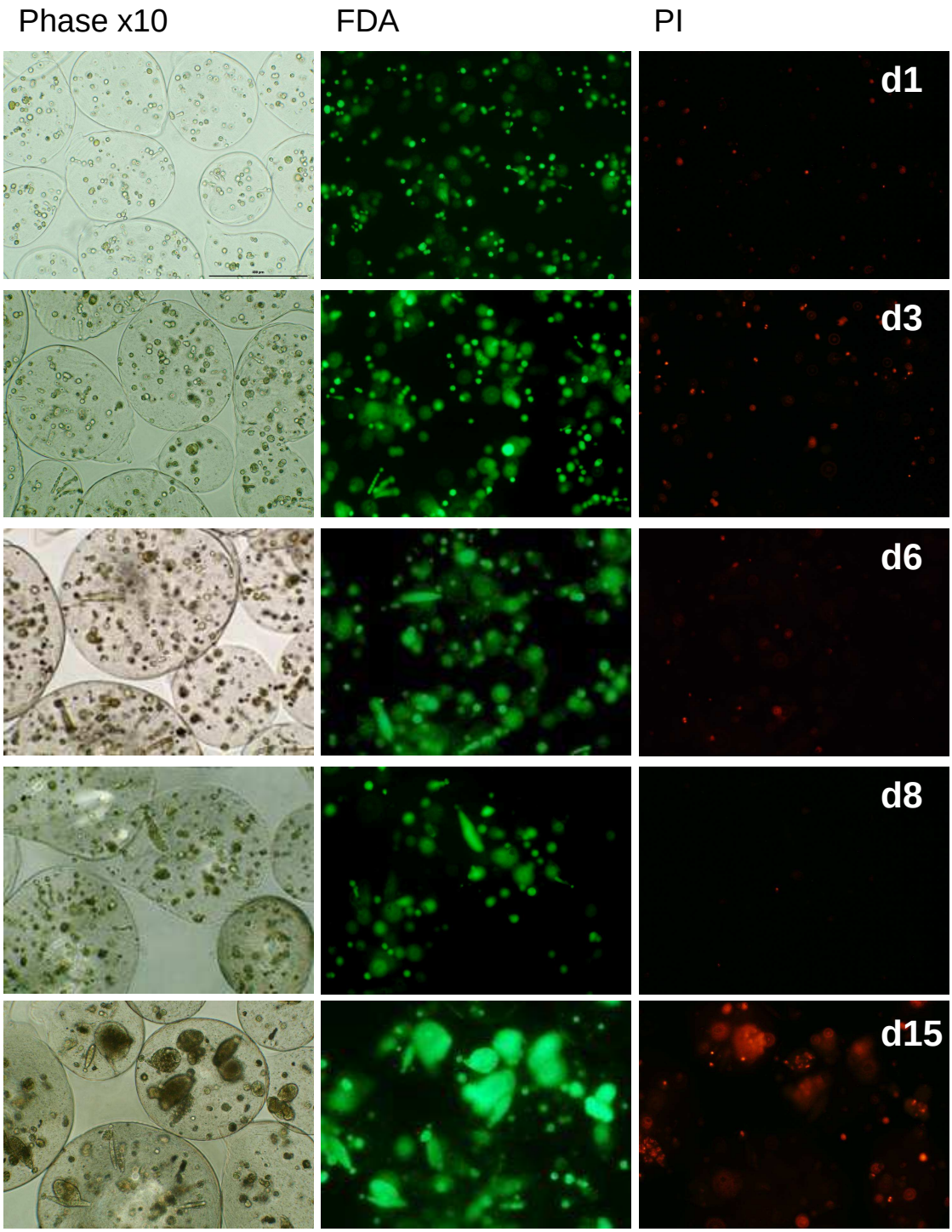


Figure 5-19. Cell viability staining (x10 magnification) of encapsulated HC-04 cells maintained in 3D static culture for up to 15 days.

5.2.2.6 CYP3A Function in HC-04 Cells is Improved in 3D-Culture

Significant effects of both culture format ($F(2, 0.017) = 40.96$, $P < 0.0001$) and chemical induction ($F(1, 0.0016) = 7.76$, $P < 0.01$) on CYP3A function (Luciferin BE metabolism) were found in HC-04 cells, and there was significant interaction between culture type and induction ($F(2, 0.0014) = 3.37$, $P < 0.05$).

Following 8 days of static culture, CYP3A function was significantly improved in alginate encapsulated HC-04 cells above monolayer culture in both vehicle (4.5 fold P<0.001) and rifampicin (5.1 fold, P<0.001) treated encapsulated cells. Following 15 days of static culture, CYP3A function remained increased relative to monolayer cultures in both vehicle (P < 0.05) and rifampicin (P < 0.05) treated cells but was significantly lower than in day 8 3D cultures following rifampicin induction (2.5 fold, P< 0.001) (Figure 5-20).

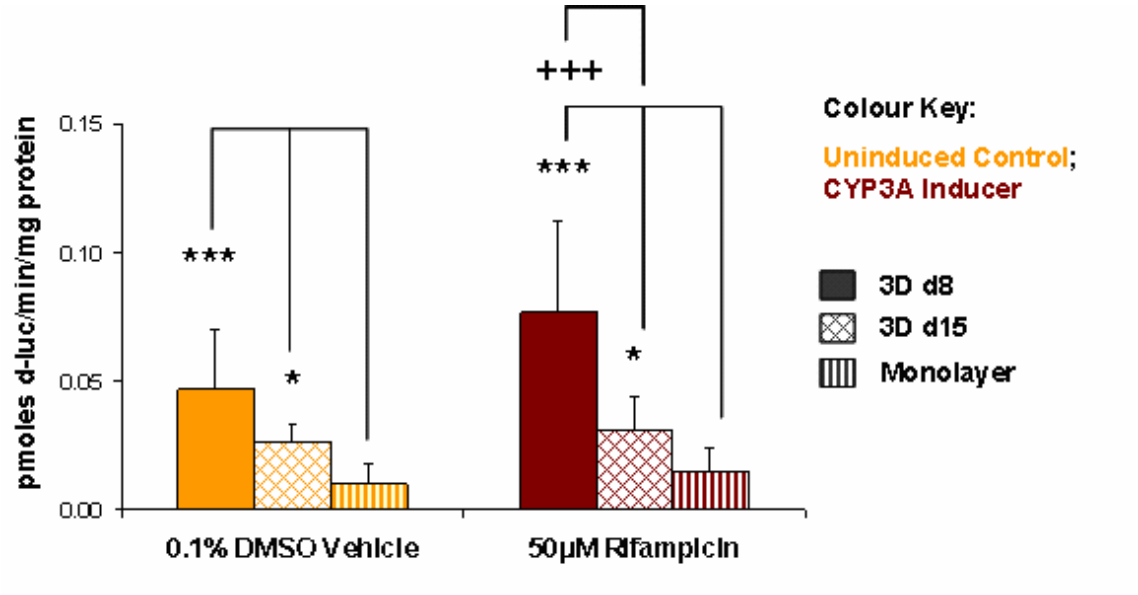


Figure 5-20. CYP3A function was measured in HC-04 cells cultured in 3D format for 8 or 15 days and treated for 48 hours with either 0.1% DMSO vehicle or 50µM rifampicin (n=6 +/- SD). Data analysis was by two-way ANOVA. ** P <0.01, *** P < 0.001 when compared to monolayer cultures, +++ P < 0.001 when d8 and d15 cultures are compared.

5.2.2.7 Influences on mRNA Expression in HC-04 Cells

Gene expression was compared in RNA isolated from HC-04 cells, maintained in either monolayer or 3D culture (8 days), and treated for 48 hours with either 0.1% DMSO vehicle or 50µM rifampicin.

5.2.2.7.1 Effect of Rifampicin Induction and 3D Culture on HC-04 CYP Induction

For each CYP isoform, significant effects of both rifampicin induction and 3D culture on CYP mRNA expression levels were observed, and there was significant interaction between the two variables. A summary of this is presented in Table 5-4.

Table 5-4. Independent and synergistic effects of rifampicin mediated induction and 3D culture on CYP expression were examined in HC-04 cells. Following two-way ANOVA, significance values P and F (degrees freedom, sum squares) are stated for each variable.

	CYP3A4	CYP3A7	CYP1A2
Induction	**P<0.05** F (1, 4.7^{-10}) = 9.876	**P<0.01** F (1, 238) = 22.10	**P<0.01** F (1, 4.1^{-10}) = 28.73
Culture Type	**P<0.01** F (1, 1.4^{-9}) = 28.77	**P<0.001** F (1, 406) = 37.75	**P<0.001** F (1, 1.0^{-9}) = 70.38
Interaction Between Variables	**P<0.05** F (1, 5.1^{-10}) = 10.59	**P<0.01** F (1, 234) = 21.76	**P<0.01** F (1, 4.1^{-10}) = 28.32

Bonferroni post tests demonstrated a significant increase in expression of each CYP gene in 3D culture, compared to monolayer, following rifampicin mediated induction (Figure 5-21). The greatest increase was measured in CYP3A7 mRNA expression (249 fold P< 0.001). Lesser increases were measured for CYP3A4 expression (27.7 fold P< 0.001) and CYP1A2 expression (16.7 fold P< 0.001).

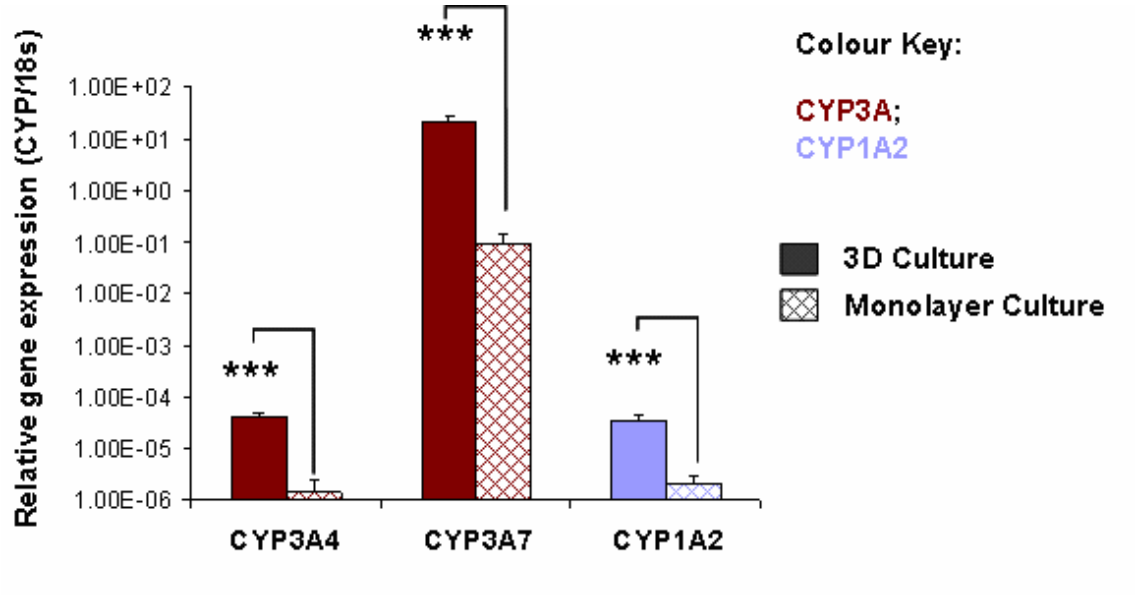


Figure 5-21. CYP mRNA expression was measured in HC-04 cells cultured in monolayer or 3D format and treated for 48 hours with 50µM rifampicin. CYP expression is normalised to ribosomal 18s expression and presented as n=3+/- SD. Following two-way ANOVA, *** P < 0.001 when monolayer and 3D cultures are compared.

5.2.2.7.2 Effect of 3D Culture on Nuclear Receptor Expression

Effects of rifampicin induction and 3D culture on gene expression were both nuclear receptor dependent, and are summarised in Table 5-5 and Figure 5-22.

PXR and RXRα expression were not significantly improved by either rifampicin induction or 3D culture. CAR expression was not influenced by rifampicin but was significantly upregulated in 3D culture ($P < 0.01$). Significant effects of both rifampicin ($P < 0.001$) and 3D culture ($P<0.001$) were observed for GR expression and there was a significant interaction between the two variables ($P<0.01$).

Table 5-5. Effects of 50μM rifampicin exposure and 3D culture on nuclear receptor expression were analysed by two-way ANOVA. Significance values P and F (degrees freedom, sum squares) are stated for each variable.

	CAR	PXR	RXRα	GR
Induction	P>0.05 $F (1, 1.8^{-27})$ = 0.007	P>0.05 $F (1, 1.8^{-7})$ = 0.97	P>0.05 $F (1, 7.6^{-20})$ =1.88	**P<0.001** $F (1, 5.7^{-15})$ =29.62
Culture Type	**P<0.01** $F (1, 3.3^{-24})$ = 12.43	P>0.05 $F (1, 5.4^{-7})$ = 2.89	P>0.05 $F (1, 1.3^{-20})$ =0.32	**P<0.001** $F (1, 1.4^{-14})$ =74.66
Interaction Between Variables	P>0.05 $F (1, 2.5^{-25})$ = 0.96	P>0.05 $F (1, 1.9^{-7})$ = 1.03	P>0.05 $F (1, 1.1^{-20})$ =0.28	**P<0.01** $F (1, 5.5^{-15})$ =28.53

Bonferroni post tests demonstrated significant increases in both CAR (21.5 fold P<0.05) and GR (104.7 fold P<0.001) expression in 3D culture compared to monolayer culture, following rifampicin exposure (Figure 5-22). There were slight increases in both PXR (2.2 fold) and RXR (2.2 fold) expression in rifampicin treated 3D cultures compared to monolayer, however, these changes were not significant.

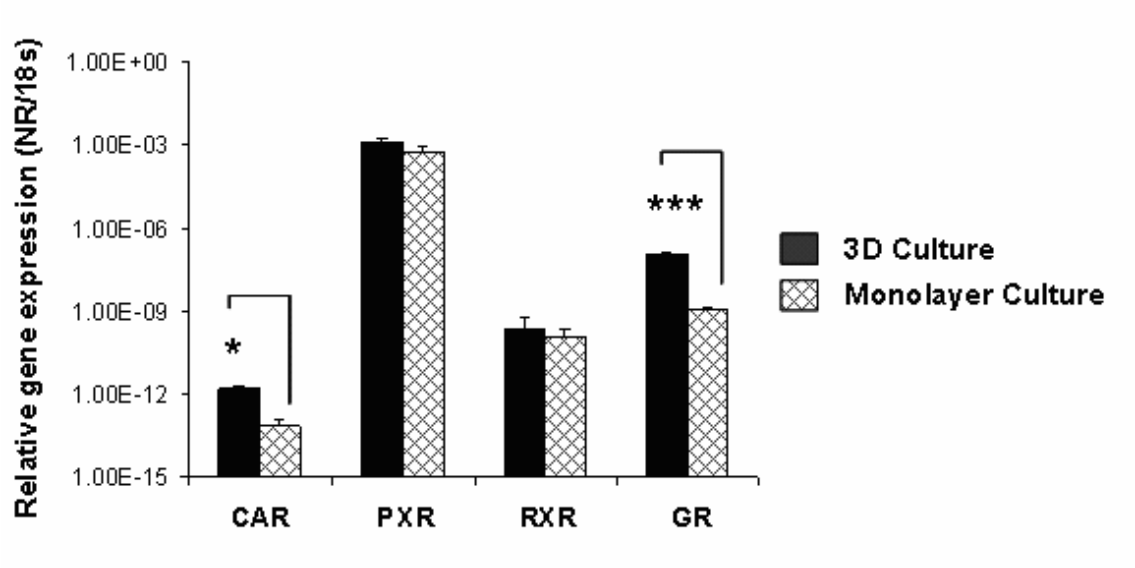


Figure 5-22. NR mRNA expression was measured in HC-04 cells cultured in monolayer or 3D format and treated for 48 hours with 50µM rifampicin. NR expression is normalised to ribosomal 18s expression and presented as n=3+/- SD. Following two-way ANOVA, * P < 0.05, *** P < 0.001 when monolayer and 3D cultures are compared.

5.2.2.8 A Comparison of PHH, HepG2 and HC-04 Cells

CYP3A activity in HC-04 cells treated with rifampicin was compared with activity measured in freshly isolated PHH (results from Chapter 3) and with HepG2 cells treated with rifampicin (results from Chapter 4). In monolayer culture, CYP3A function was comparable in HepG2 cells and HC-04 cells treated with 50µM rifampicin, but for both cell lines activity was lower than that measured in freshly isolated PHH. Following 3D culture and rifampicin treatment, CYP3A activity was higher in HC-04 cells than in HepG2 cells, and matched the average value measured in freshly isolated PHH (Table 5-6).

Table 5-6. CYP3A (Luciferin-BE) activity was higher in freshly isolated primary human hepatocytes than HC-04 cells but the difference was abrogated by 3D culture. Data was compared from PHH isolated from 9 separate donors assayed in quadruplicate and monolayer and day 8 3D cultures of HepG2 and HC-04 cells treated for 48 hours with 50µM rifampicin. Data is expressed as pmoles d-luc/min/mg protein (mean+/-SD for cell lines, mean, median and range for PHH).

Cell Type	CYP3A Activity (pmoles d-luc/min/mg protein)	
PHH Average (9 donors)	0.071	
PHH Median (9 donors)	0.051	
PHH Range (9 donors)	0.022 - 0.167	
	Monolayer	**3D (Day 8)**
HepG2 + Rifampcin	0.019 +/-0.002	0.028 +/-0.007
HC-04 + Rifampicin	0.015 +/-0.009	0.079 +/- 0.036

5.2.3 Discussion

CYP1A2 and CYP 3A induction were both demonstrated here in monolayer HC-04 cells. Significant effects of indirubin induction on CYP1A2 function were demonstrated, and improvement was shown in CYP3A function following dexamethasone and rifampicin exposure. When cultured in the same monolayer conditions, CYP3A function was comparable in HepG2 and HC-04 cells.

The beneficial effects of 3D culture on hepatocyte function are well documented and were demonstrated here for HC-04 cells with respect to their CYP function. Expression of CYP1A2, 3A4 and 3A7, as well as nuclear receptors that govern their expression, were all upregulated following alginate encapsulation. Importantly CYP3A function was significantly improved in 3D culture; following rifampicin induction CYP3A activity in d8 HC-04 spheroids matched the mean value measured in freshly isolated PHH (0.076 vs 0.071 pmoles/min/mg protein). In HC-04 cells, mRNA expression data indicates that CYP3A7 is a greater contributor to CYP3A function than CYP3A4 which

could (partly) explain (as described above for Caco2 cells) why these cells do not produce detectable quantities of the CYP3A4 testosterone metabolite 6β-OHTST [51, 215].

Interestingly, an influence of rifampicin on GR expression was also demonstrated in HC-04 cells. Induction of various NR by dexamethasone has been demonstrated in PHH and HepG2 cells [55, 96]. Furthermore, expression of GR may also influence PXR and CAR [263]. The implication of these findings would be improved CYP3A induction, which was successfully demonstrated in 3D culture, not only in HC-04 cells but also Caco2 cells.

There were drawbacks associated with alginate encapsulation of HC-04 cells. The slower proliferation of encapsulated HC-04 cells relative to both monolayer cultures and HepG2 cells would result in difficulties in obtaining an adequate biomass. Moreover, following prolonged 3D culture (which would be required for an adequate cell number), cell viability was not maintained and cell function was decreased. This phenomenon has been observed in HepG2 cells cultured in static culture and can in part be attributed to decreased oxygenation and nutrient availability and a build up of toxic waste products. This could be addressed by culturing HC-04 cells in a microgravity environment such as that provided by an RCCS or fluidised bed bioreactor (FBB). Although there are conflicting reports regarding improvement of CYP function in rotary bioreactor systems [181, 230], positive effects of RCCS function on CYP3A function were shown in Chapter 4 for HepG2 cells and at the very least, these culture methods would improve cell viability and proliferation and so, in the respect of per bead performance, would be beneficial.

Others have demonstrated that, in monolayer culture, HC-04 cells retain features that can be related to hepatocyte differentiation [160]. Albumin secretion occurs at a level comparable to PHH, additionally, low levels of ornithine transcarbamoylase and glucose-6-phosphatase are present. Arguably, slower HC-04 proliferation could be attributed to improved differentiation, which occurs in 3D culture through superior polarity and cell-cell interaction, however, this could only be proved by attempting to enhance HC-04 growth in 3D format and then re-examining CYP3A function.

5.3 Conclusions

In this Chapter, alternative cell sources that could provide a source of CYP3A mediated drug metabolism within a BAL were investigated.

Inducible CY3A function was demonstrated in Caco2 cells and this function was significantly enhanced in 3D culture following rifampicin induction, however, this function was still 3 fold less than the lowest level measured in PHH. Additionally, Caco2 cells were unable to metabolise testosterone in an equivalent manner to PHH.

Inducible CYP3A function was demonstrated in LS147T cells, and it was higher overall in these cells than in Caco2 cells. CYP3A activity was also significantly improved in LS147T cells by 3D culture; following prolonged exposure to VD3, LS147T cells produced the CYP3A4 testosterone metabolite 6β-OHTST and Luciferin BE activity was within the upper quartile limits of activity measured in PHH. Unfortunately there were several practical reasons why LS147T cells would not be suitable for BAL use. Firstly, CYP3A function comparable to PHH was only achieved after prolonged VD3 exposure. VD3 is a relatively expensive compound, and this would be a prohibitive factor for large scale BAL culture. Secondly, LS147T cells in monolayer culture produce a large amount of debris; this was not improved in 3D culture and would present practical difficulties when fulfilling regulatory requirements for a BAL, which include complete removal of DNA contamination from the biomass before plasma is returned to the patient.

HC-04 cells, as an alternative hepatic cell line for a BAL, could provide inducible CYP3A function. This function remains stable over multiple passages, but not at sufficient levels in monolayer culture. It was demonstrated here that alginate encapsulated HC-04 cells can be successfully cultured over a prolonged period of time (up to 15 days) and although further work would be required to optimise the growth and performance of these cells within a 3D culture system, the functional data obtained thus far is promising: following rifampicin induction Luciferin BE activity in d8 HC-04 spheroids matched the mean value measured in freshly isolated PHH. Additionally, functional data obtained in monolayer culture together with mRNA expression data indicate that HC-04 cells would be able to provide CYP1A2 metabolism at sufficient levels also. Limitations of HC-04 cells with respect to their use in a BAL include their poor testosterone metabolism relative to PHH and their rate of proliferation in 3D

culture, which was decreased relative to HepG2 cells even when cells were encapsulated at double the seeding density. Also, as mentioned previously, these cells were initially contaminated with mycoplamsa, which could result in difficulties fulfilling regulatory requirements surrounding the original source of a cell line suitable for use in a device to be used to treat humans.

Although HepG2 cells have diminished CYP function and limited CYP3A induction, many other factors, including the achievable biomass size and synthetic capacity of these cells, mean that they remain an attractive option for a BAL. A genetic manipulation approach has been used successfully to restore urea cycle function to HepG2 cells [13], and this may be an option for achieving drug metabolism capacity.

It has been demonstrated that CYP mRNAs including CYP3A are expressed in HepG2 cells, albeit at a lower level than in PHH; methods that improve existing CYP expression could result in increased CYP3A function within these cells. Other groups have demonstrated the potential for CYP3A function in HepG2 cells, often by transfection of single CYP isoforms [142-144]. Methods by which the function of multiple CYP isoforms can be improved in HepG2 cells by genetic manipulation will be investigated in Chapter 6.

CHAPTER 6 Modifying CYP3A Function in HepG2 Cells

As described in Chapter 4, HepG2 cells can be manipulated to perform CYP mediated functions and would provide CYP1A function within a BAL; however, even though significantly improved by 3D culture (relative to monolayer), only limited CYP3A function can be achieved. CYP1A activity alone is insufficient for a BAL since in humans it accounts for only 10% of xenobiotic metabolism and may result in procarcinogen production [42, 264].

Alternative cell lines to provide CYP3A activity were described in Chapter 5. Intestinal cell lines had inducible CYP3A4 function that was upregulated by 3D culture and induction, however, in the case of Caco2 cells, CYP3A activity was insufficient. LS147T cells had relatively greater CYP3A4 function but were unsuitable for BAL culture. Also, intestinal cell lines within a BAL could only be used to provide detoxificatory capacity and would not perform other hepatocyte specific functions. HC-04 cells were therefore assessed as an alternative liver-derived cell line. They demonstrated CYP3A activity which was significantly enhanced by 3D function, however their growth in 3D culture was relatively slow which could present a problem obtaining an adequate biomass. There were also regulatory difficulties associated with this cell line.

In spite of their limited detoxificatory capacity, HepG2 cells remain an attractive candidate for BAL use. These cells have been well characterised within the Liver Group (LG) BAL [135] and perform many hepatocyte specific functions; furthermore a subclone of these cells have been used in clinical trials which sets a precedent for regulatory approval [131].

The aim of Chapter 6 is to improve, by genetic manipulation, CYP3A function in HepG2 cells. Further to this, an alternative source of HepG2 cells will be considered, as will the effects of adaptation of these cells to LG αMEM complete medium.

6.1 Improving Inducible CYP3A Function in HepG2 Cells

Decreased mRNA expression of CYPs provides one explanation for the low functional activities of these enzymes in hepatoma cells [45]. CYP3A4 transcripts are present in HepG2 cells although mRNA levels are far lower (100-1000 fold) than in adult primary human hepatocytes [136]. It is therefore a reasonable hypothesis that improving expression of CYP mRNA could improve enzyme function. Other laboratories have previously engineered HepG2 cell lines which stably express single CYP isoforms [143, 144] but sustained function of multiple CYP isoforms, at a high level, through this approach is difficult to achieve.

As described in Chapter 1, CYP induction occurs by ligand activated nuclear receptors (NR) forming heterodimers with a co-receptor leading to increased CYP gene transcription. CYP3A induction is governed by a heterodimer of Pregnane X Receptor (PXR) or Constitutive Androstane Receptor (CAR) with Retinoid X Receptor alpha (RXRα) [30, 77, 265, 266]. These heterodimers bind to response elements (RE) in the CYP3A gene increasing CYP3A transcription resulting in enhanced enzyme production. Others have established that altering HepG2 NR expression can improve CYP3A transcription [147, 150].

Inducers may act directly at nuclear receptors (rifampicin at PXR), indirectly (phenobarbital causes translocation of CAR from cytoplasm to nucleus following receptor dephosphorylation) [267], and synergistically (dexamethasone not only directly activates PXR, but can mediate the expression of RXRα, PXR and CAR via glucocorticoid receptor activation) [96].
CAR expression is diminished in HepG2 cells relative to primary human hepatocytes [157, 221], and expression levels vary throughout the cell cycle [268], which may contribute to lower expression of CYP3A in these cells. RXRα mRNA expression is decreased in HepG2 cells relative to other human hepatoma cell lines [232]; additionally, in certain circumstances, ligands binding to RXRα may reduce the availability of this receptor for CAR, repressing CYP gene expression [269, 270]. It is therefore hypothesised that transient transfection of the nuclear receptor CAR and/or co-receptor RXRα in conjunction with chemical induction could improve CYP function in HepG2 cells to provide function comparable to that in primary human hepatocytes.

Each CYP gene contains multiple response elements which may be activated by more than one nuclear receptor. Multiple CYP enzymes may be induced by a single inducing agent or alternatively, a single CYP gene may be activated by more than one inducing agent. CAR is primarily reported to be an inducer of CYP2B6, but activation of CAR also results in increased CYP3A transcription and it has been implicated in the induction of other isoforms including CYP1A [231, 271-273]. Due to this receptor cross-talk/non-selectivity of nuclear receptor induction pathways, a single transfectant might result in improved function of multiple CYP isoforms resulting in the creation of a BAL relevant cell line that can provide xenobiotic metabolism. A sub-hypothesis is that co-transfecting combinations of nuclear receptors or associated co-receptors will result in increased inducible CYP3A function.

The data here characterises CYP1A2 and CYP3A function in HepG2 cells following RXRα and/or CAR transfection. Proof of concept was achieved in HepG2 cells in a transient system by measuring CYP functional activity with and without inducers. This work consisted of the following.

i.) Creating Constructs Expressing CAR and RXRα
   a.) Designing cloning primers for nuclear receptor sequences
   b.) Amplifying nuclear receptor sequences from human liver cDNA
   c.) Inserting amplified sequences into pTracer EF/BSD

ii.) CYP Induction in HepG2 Cells Transiently Expressing DNA Constructs
   a.) Transiently transfecting HepG2 cells with nuclear receptor constructs
   b.) Treating transfectants with CYP inducers
   c.) Measuring CYP function

iii.) CYP Function of CAR Transfectants in 3D Culture
   a.) Creation of stable single transfectant
   b.) Measurement of function in monolayer culture
   c.) Measurement of function in 3D culture

6.1.1 Creation of Constructs

6.1.1.1 Methods

6.1.1.1.1 Designing Primers for Cloning

Oligocalc (http://www.basic.northwestern.edu/biotools/oligocalc.html) software was used to design primers which were BLAST screened for specificity.

For CAR (NM_001077482) and RXRα (NM_002957), a nested PCR approach was selected. Full Genbank sequences were obtained for each nuclear receptor. Forward and reverse outer primers were designed ~ 200bp upstream and downstream of the full cDNA sequence to obtain a PCR product that contained the full sequence for these nuclear receptors.

Cloning primers were designed to amplify the full cDNA sequence of each nuclear receptor. A Kozak initiation sequence was added to the forward primers; KPN I (RXRα) or EcoRV (CAR) restriction sites were added to the beginning of the forward primers and an XbaI restriction site added to the ends of the reverse primers (Table 6-1).

Table 6-1. Nested PCR primers for amplification of RXR*α* and CAR listed in 5'-3' orientation. Restriction sites and a *Kozak sequence* were incorporated into Cloning Primers.

		RXRα
Outer Primers	*Forward*	GGCATGAGTTAGTCGCAG
	Reverse	GCCACAGACAAGTAGTAAC
Cloning Primers	*Forward*	GA**GGTACC**C*GCCACC* ATGGACACCAAACATTTC
	Reverse	GGGCC**TCTAGA**CCTAAGTCATTTGGTG

		CAR
Outer Primers	*Forward*	CAGCCACCCCAACACGTGAC
	Reverse	GGTGAGCTGGGGAAGGAAGT
Cloning Primers	*Forward*	CAC**GATATC**ACGTG*CCACC* ATGGCCAGTAGGGAAGATG
	Reverse	GGAACGA**TCTAGA**ATGGCCTCAGCTGCAGATCTCCTGGA

6.1.1.1.2 Amplifying Nuclear Receptors from Human Liver

High-fidelity DNA polymerase was used to amplify sequences from human liver cDNA which was synthesised from RNA isolated from human liver using the methods described in Chapter 2.

Materials

Human Liver cDNA

Phusion High-Fidelity DNA Polymerase, HF buffer and accompanying reagents (NEB F5305)

10µM Outer Primers (OP)

10µM Cloning Primers (CP)

Sterile Water

Montage DNA extraction Columns

Methods

For each set of outer primers, PCR reactions were set up in triplicate as follows:

Reagent	**µl/20µl Reaction**
H_20	11.4
5x HF Buffer	4
10mM dNTP	0.4
Forward Primer	1.2
Reverse Primer	1.2
DMSO	0.6
Template	1
Phusion	0.2

PCR conditions were: 98°C for 30s for initial denaturation, followed by 30 cycles of 10s at 98°C denaturation, 30s at 60°C annealing and 60s at 72°C extension, with a final extension step of 10 minutes at 72°C. Product was then run on a 1% agarose gel, and DNA extracted (using Montage DNA Gel Extraction Columns), purified and concentrated by ethanol precipitation as described in Chapter 2. Purified PCR product or a diluted sample (1:20 or 1:200) of the original PCR product was loaded into a subsequent reaction with cloning primers to add restriction sites. Product was run on a 1% agarose gel, and extracted, concentrated and purified for subsequent steps as described above.

6.1.1.1.3 Inserting Sequences into pTracer EF/BSD Vector

pTracer™-EF/Bsd A contains a cycle 3-GFP (green fluorescent protein) tag fused to a blasticidin resistance gene. The GFP in this vector has been optimised for expression in mammalian cells and gives a greater fluorescent yield than the wild type. This allows transfection efficiency and subsequent gene expression to be easily monitored by fluorescence microscopy and the fused blasticidin resistance gene allows selection in mammalian cell lines which can be directly correlated with GFP expression. Gene expression in this vector is driven by human elongation factor 1α-subunit (hEF-1α) promoter (Figure 6-1).

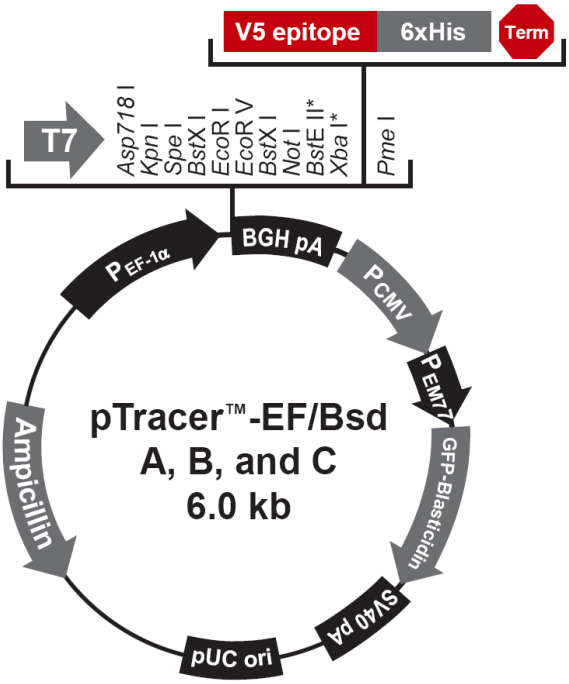


Figure 6-1. Configuration of pTracer EF/BSD A (Invitrogen V889-20 Version C May 2003)

6.1.1.1.4 Restriction Digests of Vector and Insert

Restriction Enzymes (NEB: Eco RV, Kpn I, Xba I)

NEB Buffer 2 (10x concentration)

10mg/ml BSA (NEB 10x concentration)

pTracer™-EF/Bsd A (Invitrogen V889-20)

DNA purified from Phusion PCR (CAR, RXRα)

Sterile water

Kpn I and XbaI were used to digest RXRα DNA; CAR DNA was digested using EcoRV and XbaI; and the vector was digested with both sets of restriction enzymes. For each restriction digest, the following reaction was prepared in a 0.2ml PCR tube:

1µg DNA (typically 5-10µl)
1.5µl Buffer 2
1.5µl BSA
0.5µl EcoRV or Kpn I
0.5µl XbaI
Sterile water to a final volume of 15µl

Tubes were incubated at 37°C for 2 hours. Digestion was confirmed by running 2µl of product on a 1% agarose gel. The remainder of each reaction was then concentrated by ethanol precipitation as described above.

6.1.1.1.5 Ligation of Vectors and DNA

NEB Quick Ligase was used to ligate vector and insert. For each reaction a control ligation containing vector without insert was also performed.

Materials
Digested vector and insert
NEB Quick Ligase Kit (M2200)
Sterile water
0.2ml PCR tubes

A 3:1 ratio (ng/ng) of digested insert and corresponding vector were combined in a volume of 4µl in 0.2ml PCR tubes. To this mix, 5µlof 2x Quick Ligase buffer was added followed by 1µl Quick Ligase. The reaction was mixed by pipetting and then incubated at 25°C for 1 hour.

6.1.1.1.6 Transforming Competent Cells (E. coli) with Constructs

XL10-Gold Ultracompetent Cells (Stratagene 200315)

β-Mercaptoethanol

Ligation Mix

SOC Broth pH 7.0 (*for1L: 0.5g NaCl, 5g yeast extract, 20g tryptone, sterile distilled H_2O to 950ml, add 10ml 250mM KCl, autoclave autoclave 121°C, 20 minutes, add 20ml filter sterilized 1M glucose and 5ml autoclaved 2M $MgCl_2$*)

LB Agar containing 100µg/ml carbenicillin (*for 1L: 5g NaCl, 2.5g yeast extract, 5g tryptone, 10g agar, pH7.0 with 5M NaOH autoclave 121°C, 20 minutes and then add the antibiotic when cooled sufficiently*)

42°C Water bath

37°C Shaker

37°C Incubator

LB Agar plates were poured and dried upside down in a 37°C incubator. For the transformation, the manufacturer's protocol was followed. XL10-Gold Ultracompetent cells were thawed on ice, 4µl of β-mercaptoethanol added and mixed every 2 minutes, by gentle swirling, for 10 minutes. After this time, 40µl aliquots of the cells were transferred into sterile microfuge tubes, 4µl of ligation mix added and the mixture incubated on ice for 30 minutes. Tubes were heat pulsed in a 42°C water bath for 30 seconds, returned to ice for 2 minutes and then 400µl of warmed SOC broth added. Samples were incubated at 37°C for 1 hour with shaking at 225rpm. 200µl of transformation mix was then spread onto the prepared agar plates and incubated overnight at 37°C.

6.1.1.1.7 Identifying Positive Colonies

Positive transformation was confirmed by the growth of discreet white colonies on the agar plates. The control ligation was used to indicate transformation efficiency of the empty vector. Primers which spanned the multiple cloning site (MCS) of the vector were then used to screen selected colonies.

A PCR was set up using HotstarTaq, as described in Chapter 2.

Primers (5'-3'orientation):

Forward (EF-1α priming site): TCA AGC CTC AGA CAG TGG TTC

Reverse (BGH priming site): TAG AAG GCA CAG TCG AGG

Cycling conditions:

95°C	10 minutes	
95°C	30s	30x cycles
55°C	30s	
72°C	90s	
72°C	10 minutes	

PCR mix (20µl) was aliquoted into 0.2µl PCR tubes. A selection of colonies, usually 10-20 depending on transformation efficiency were picked individually and streaked onto fresh LB agar plates using a 20µl pipette tip. The used tip was then inserted into the PCR mix. The PCR was performed as indicated above and product run on a 1% agarose gel.

6.1.1.1.8 Miniprep of *E.coli* Containing Constructs

LB broth containing 100µg/ml carbenicillin (*for 1L LB 5g NaCl, 2.5g yeast extract, 5g tryptone, pH7.0 with 5M NaOH autoclave 121°C 20 minutes*)

37°C shaker

50ml tubes

Miniprep plasmid extraction kit (Qiagen 27106)

Sterile Glycerol

1.5ml and 2ml microfuge tubes

Micro centrifuge

NanoDrop

Positive colonies, as shown by the MCS PCR were picked off the streaked plates. Each colony was used to inoculate 5ml of LB broth which was incubated overnight (but less than 17 hours) at 37°C in a shaker. The following day, 2ml of each culture, in duplicate, was centrifuged at 16000g for 1 minute. From the remaining culture a 15% (v/v) glycerol stock was prepared which was stored at -80°C until required. The construct was isolated from the pelleted culture mix according to the miniprep plasmid extraction kit protocol. The amount of DNA obtained was quantified using the NanoDrop.

Restriction digests were performed for each construct as described in section 6.1.1.1.4. to confirm that both vector and insert were of the desired product size. 300ng of each construct was sent to the UCL Facility for Sequencing and Genotyping to be sequenced.

6.1.1.2 Results

6.1.1.2.1 Cloning of CAR and RXRα from Human Liver cDNA

The amplification of CAR from human liver and analysis of colonies obtained when DNA was inserted into pTracer vector are shown in Figure 6-2. The coding region of CAR is 1074bp and primers that spanned the MCS gave a PCR product of 1362bp.

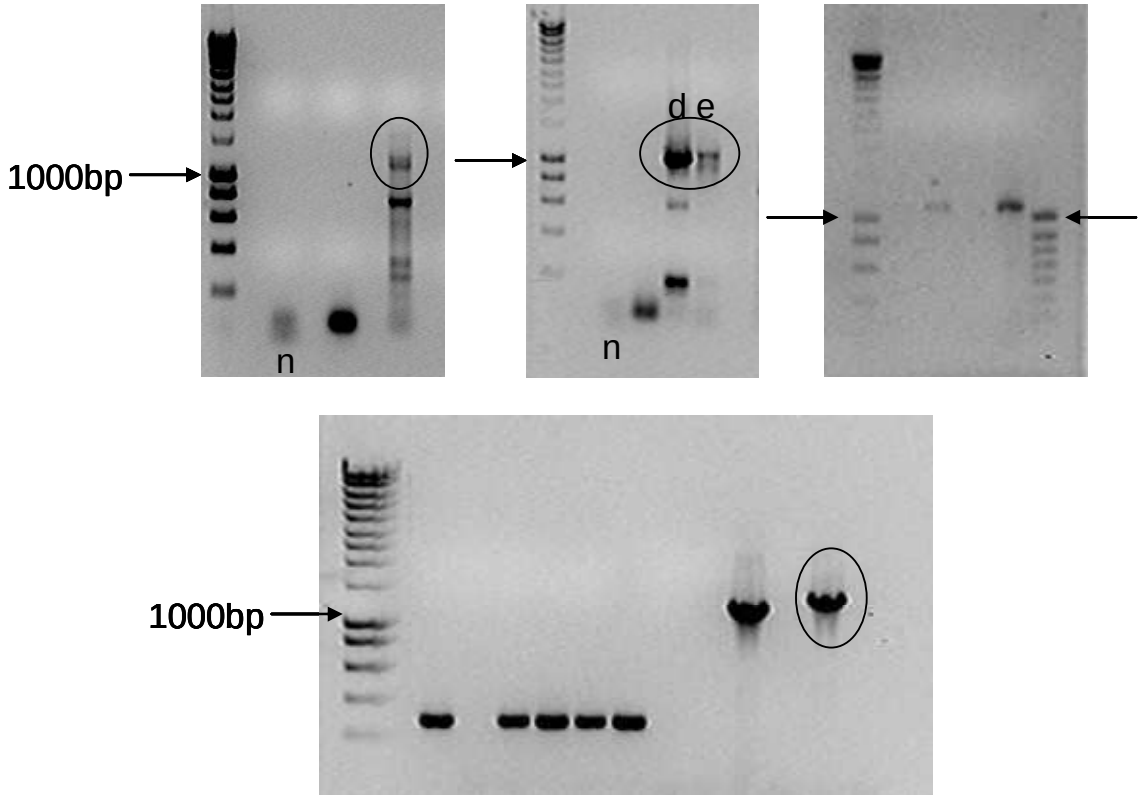


Figure 6-2. Cloning of CAR from human liver DNA.

The top gels show nested PCR amplification of CAR from human liver cDNA. Arrows indicate 1000 bp markers.

(top left) Product was first amplified with outer primers and the circled band excised from gel and purified. (top middle) Using cloning primers to add restriction sites, a second PCR was prepared to amplify CAR from PCR product from the first reaction that had (d) been diluted 1:200 or (e) extracted from the gel on the left. No template controls are marked n. Circled bands were then excised from the gel and purified by ethanol precipitation to give a product of 1074bp (top right).

The bottom gel shows MCS PCR of transformed colonies for which the desired product size was 1362 bp. The circled product was used for subsequent transfections following sequencing.

The amplification of RXRα from human liver and analysis of colonies obtained when DNA was inserted into pTracer vector are shown in Figure 6-3. The coding region of RXRα is 1389bp and primers that spanned the MCS gave a PCR product of 1635bp.

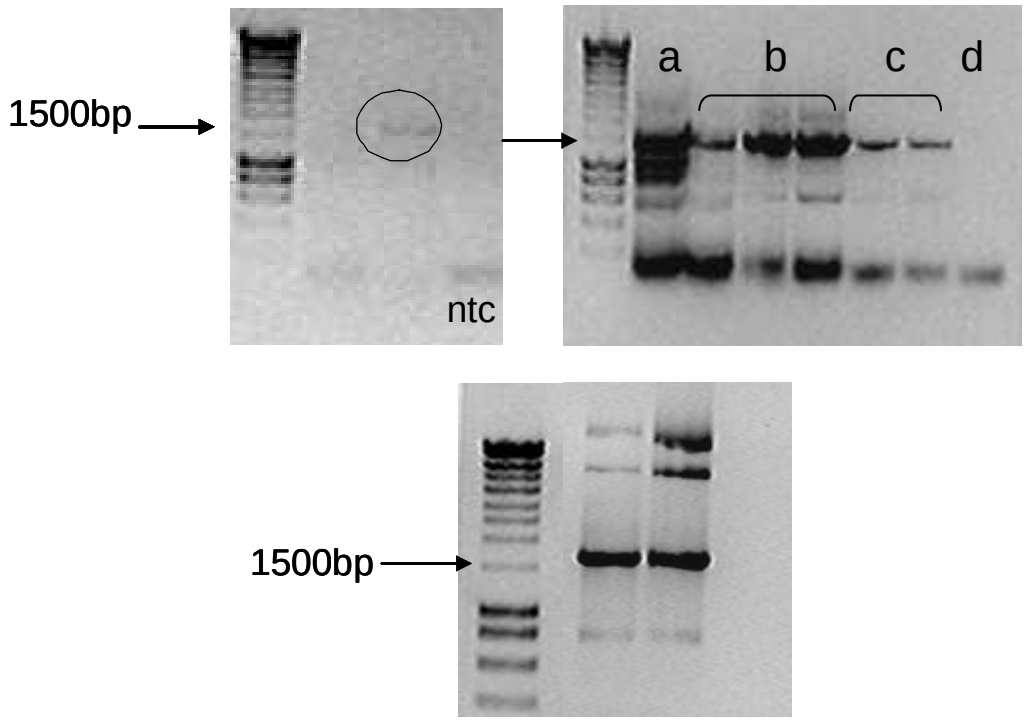


Figure 6-3. Cloning of RXRα from human liver DNA.

The top gels show nested PCR amplification of RXRα from human liver cDNA.

(top left) Product was first amplified with outer primers and the circled band excised from gel and purified. (top middle) Using cloning primers to add restriction sites, a second PCR was prepared to amplify RXRα from PCR product from the first reaction that had been diluted (a) 1:20, (b) 1: 200 or (c) been extracted from the gel on the left. No template control is indicated (d). Bands from (c) were then excised from the gel and purified by ethanol precipitation to give a product of 1389bp.

The bottom gel shows MCS PCR of transformed colonies following miniprep for which the desired product size was 1635 bp. This product was used for subsequent transfections following sequencing.

6.1.2 Transient Transfection of Nuclear Receptors and CYP Induction

6.1.2.1 Methods

6.1.2.1.1 Transient Transfection and CYP Induction in HepG2 Cells

A reverse transfection method was used to transfect cells using Lipofectamine™ 2000 reagent (Invitrogen UK) according to manufacturer's instructions. HepG2 cells ($1x10^5$) were added to each well of a 96 well plate along with transfection mix containing 0.2µg of DNA construct and 0.5µl of lipofectamine, or lipofectamine alone as a mock

transfection control. Following an overnight incubation, serum free medium without antibiotics was replaced with normal growth medium (αMEM Complete 10%FCS) containing CYP inducers. Prior to measurement of CYP function, cells were incubated for 48 hours in the presence of inducers, and medium replenished daily. CYP function was measured by Luciferase and Vivid® BOMFC assays. Cell viability was measured by WST-1 assay. Protein content was determined by the BCA assay. Each experiment was repeated three times in triplicate and data are presented as mean of 9 values +/- SEM.

6.1.2.1.2 Gene Expression in Transfected HepG2 Cells

Gene expression studies (mRNA) were carried out at 72 hours post transfection. RNA was isolated from transfected HepG2 cells and gene expression was performed by qRT-PCR as described in Chapter 2.

6.1.2.2 Results

6.1.2.2.1 Transfection Efficiency in HepG2 Cells

Transfection efficiency was monitored by GFP expression in the NR transfected cells (Figure 6-4).

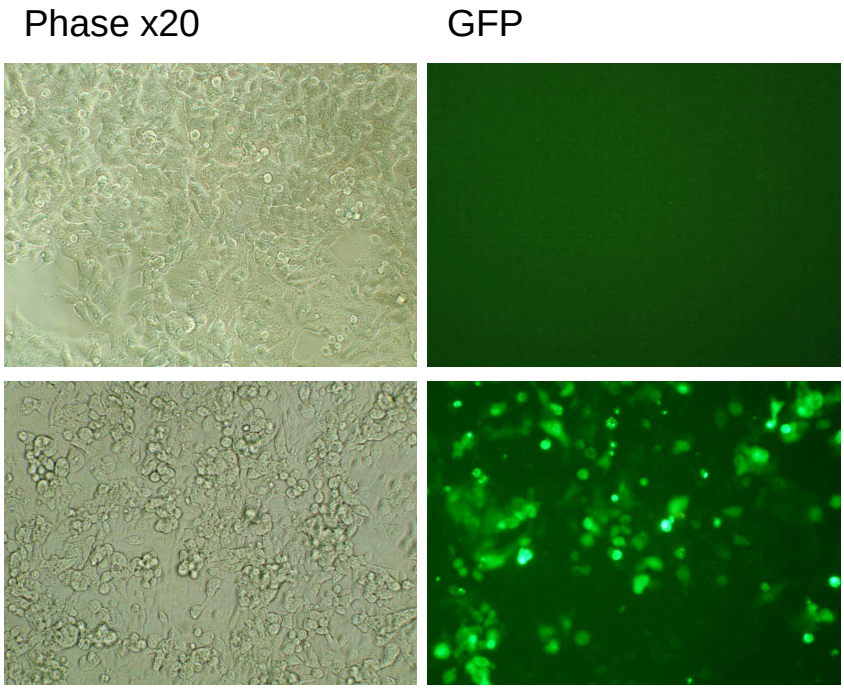


Figure 6-4. Transfection efficiency was monitored by comparing GFP expression in (top) untransfected HepG2 cells and (bottom) HepG2 cells 96 hours post transfection. Images are x20 magnification.

Increased transgene expression was confirmed 72 hours post transfection by qRT-PCR. Gene expression was compared in HepG2 cells and human liver pooled from 4 different donors and although gene expression of both RXRα and CAR were diminished in HepG2 cells, it was restored to levels similar to those measured in human hepatocytes by transient transfection (Figure 6-5).

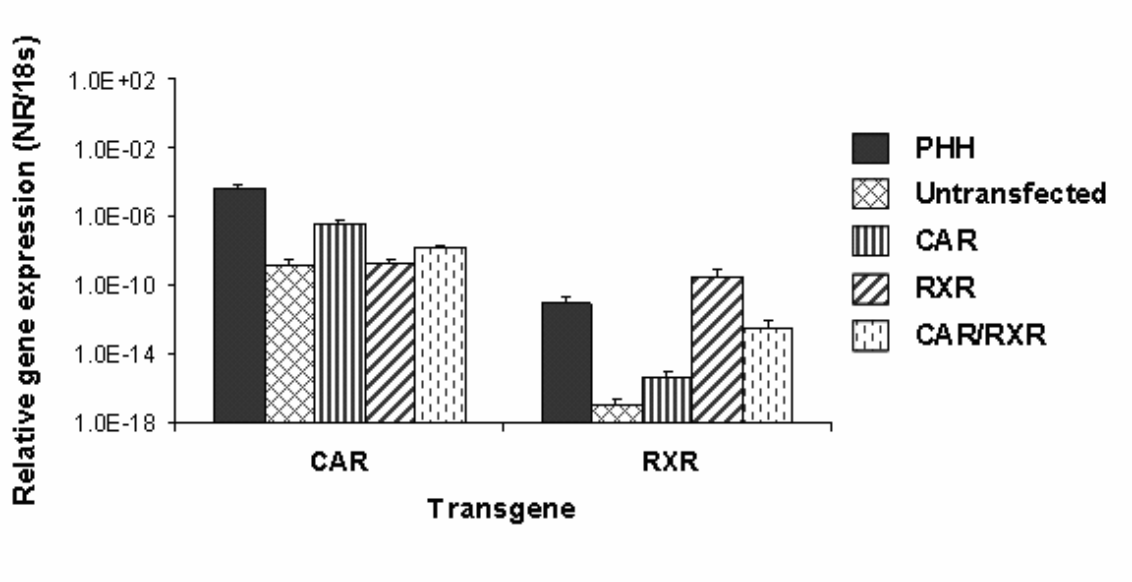


Figure 6-5. HepG2 cell CAR and RXRα expression was determined by RT-qPCR in mRNA isolated 72 hours post transfection from control transfected cells and cells transfected with CAR, RXRα and CAR/RXRα. mRNA was included from freshly isolated PHH cells pooled from 4 separate donors as a comparator. Data was normalised to 18s expression and is expressed as mean (n=2) +/- range (repeated twice).

6.1.2.2.2 Effect of Transfection on CYP3A (Luciferin BE) Activity

Data was analysed by two-way ANOVA and significant effects of both gene transfection ($F(3, 196) = 16.03$, $P < 0.0001$) and chemical induction ($F(6, 196) = 7.60$, $P < 0.0001$) were measured on CYP3A function in HepG2 cells, however no interaction was observed between the two variables. In their uninduced state, Luciferin BE metabolism was somewhat higher in cells transfected with CAR/RXRα (1.5-fold) and to a lesser extent with CAR (1.2-fold), than in untransfected HepG2 cells but the changes were not significant.

As shown in Figure 6-6, for each inducer, the fold induction above basal achieved was equivalent for each transfectant (although a trend towards increased induction is indicated for the CAR transfectant).

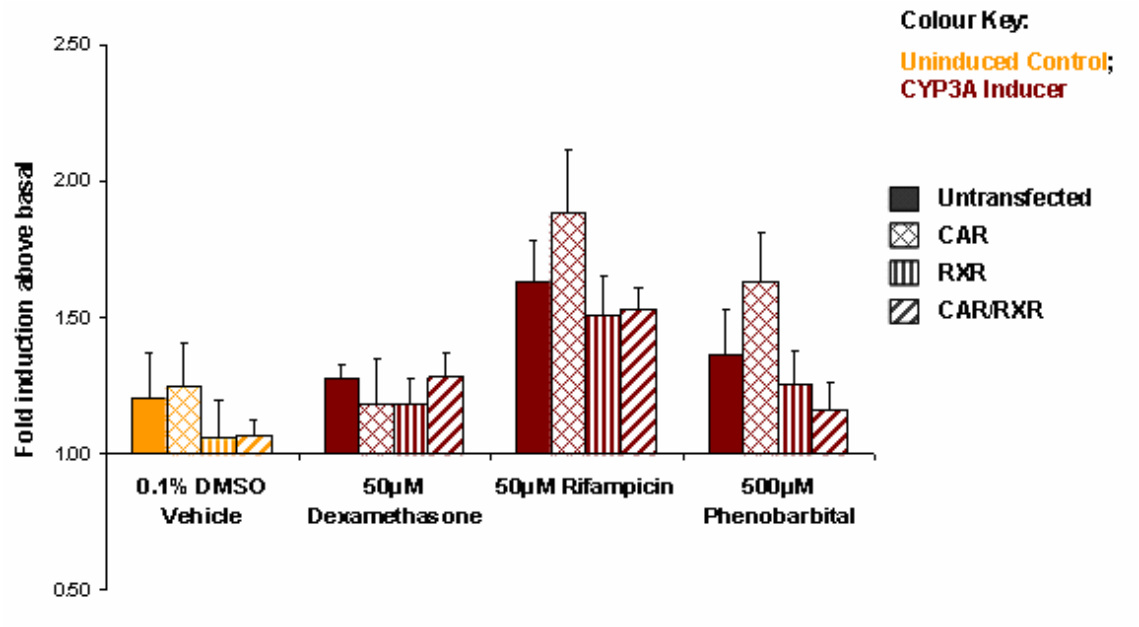


Figure 6-6. CYP3A induction in HepG2 cells following 48 hour treatment with CYP3A inducers was measured by Luciferin BE activity in HepG2 cells following transfection with either mock control, CAR, RXRα or CAR/RXRα. For each transfectant, basal (uninduced) activity was set at 1 and induction was measured above this level.

Transfected HepG2 cells had a higher basal activity. Since this study was concerned with achieving a cell line with high inducible activity, the fold increase in activity above untransfected uninduced HepG2 cells was then examined for each transfectant to measure which inducer/transfectant combination resulted in the most significant increase in CYP3A function above this level.

6.1.2.2.3 Effect of Transfection on CYP3A (Luciferin BE) Activity Following Treatment with CYP3A Inducers (Figure 6-7)

In untransfected HepG2 cells, there was slight induction of CYP3A function above uninduced cells using rifampicin (1.6-fold), dexamethasone (1.3-fold) and Phenobarbital (1.4-fold) but these changes were not statistically significant.
After CAR transfection, rifampicin induced CYP3A activity was enhanced (2.1-fold, $P<0.05$). This was further improved by RXRα co-transfection (2.3-fold, $P<0.001$).

After CAR transfection, phenobarbital induced CYP3A activity was increased (1.9-fold); this was not improved by RXRα co-transfection.

Dexamethasone mediated induction was not enhanced by RXRα or CAR transfection alone, however, co-transfection of these genes led to a 1.9-fold induction in CYP3A activity compared to uninduced untransfected HepG2 cells.

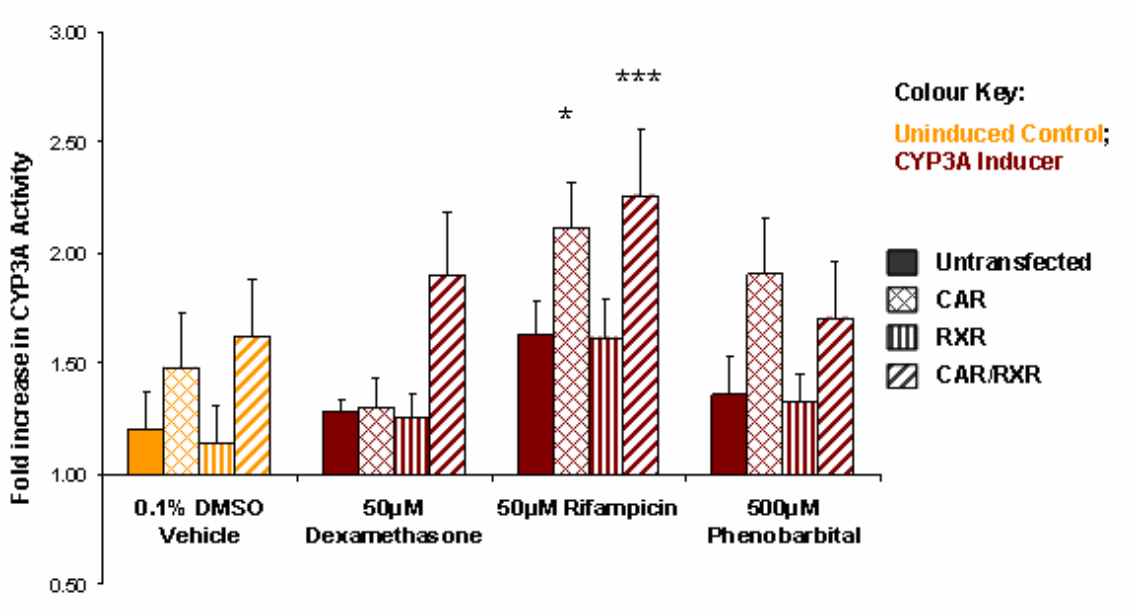


Figure 6-7. CYP3A activity was measured in HepG2 cells following transfection with CAR, RXRα, CAR/RXRα or mock control and 48 hour treatment with CYP3A inducers. CYP3A activity in untransfected uninduced cells was set as 1 and data is expressed as fold increase in activity compared to this. Following one way ANOVA, * P<0.05, *** P< 0.001 when compared to untransfected uninduced HepG2 cells.

6.1.2.2.4 Effect of Transfection on CYP3A (Luciferin BE) Activity Following Treatment with CYP1A Inducers

Effect of AhR agonists (CYP1A inducers) on CYP3A activity is shown in Figure 6-8. In untransfected cells, CYP3A function was increased above basal in cells treated with both DBA (1.5-fold) and indirubin (1.8 fold).

The increased basal activity, after CAR and CAR/RXRα transfection, improved activity relative to untransfected cells following treatment with either inducer. This resulted in a significant increase in CYP3A activity above untransfected uninduced cells following indirubin treatment of CAR/RXRα transfected cells (2.3 -fold, P<0.001).

Transfection of RXRα alone had a limiting effect on Luciferin BE metabolism following treatment with both DBA and indirubin. When measured against untransfected uninduced cells, the extent of indirubin mediated CYP3A induction was

significantly reduced in RXRα transfected cells (1.2-fold) compared to CAR/RXRα transfected cells (P<0.05).

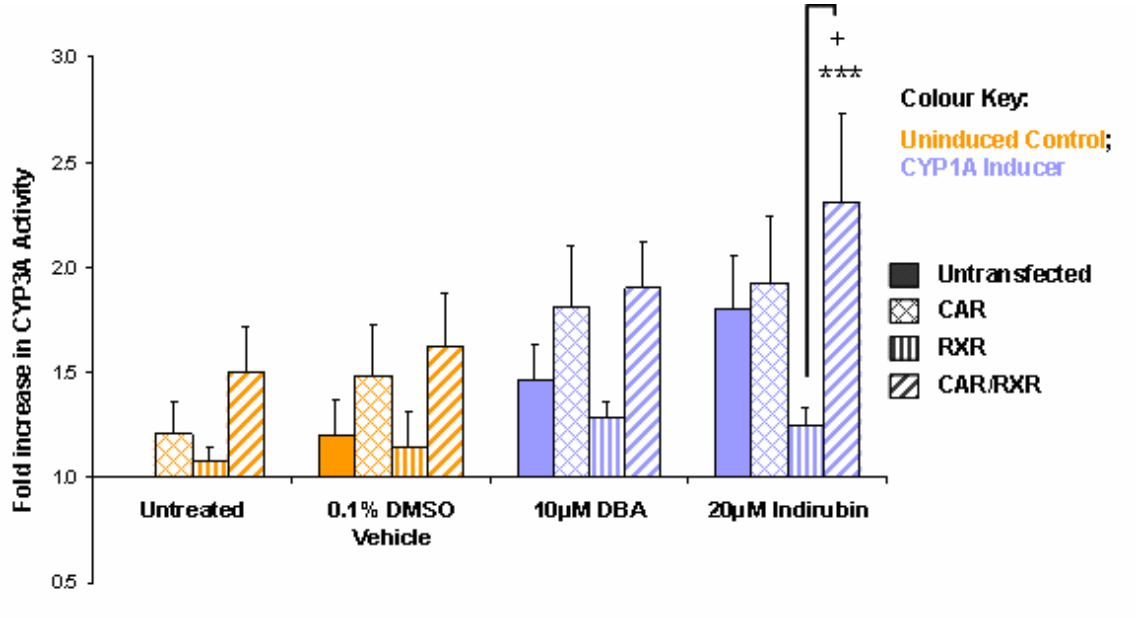


Figure 6-8. Effect of AhR agonists on CYP3A activity was measured in HepG2 cells transfected with mock control, CAR, RXRα or CAR/RXRα and treated for 48 hours with inducers to assess receptor cross-talk. CYP3A activity in untransfected uninduced cells was set as 1 and data is expressed as fold increase in activity compared to this. Following one way ANOVA, *** P< 0.001 when compared to untransfected uninduced cells. + P<0.05 when RXRα transfectant was compared with CAR/RXRα co-transfectant.

6.1.2.2.5 Effect of Transfection and Induction on CYP1A2 (Luciferin ME) Activity

Luciferin ME assay was performed in the presence of 10µM sulfaphenazole. Significant effects of both gene transfection ($F(3, 188) = 13.86$, $P < 0.0001$) and chemical induction ($F(6, 188) = 53.87$, $P < 0.0001$) on CYP1A2 function were found in HepG2 cells, with a significant interaction between transfection and induction ($F(18, (188) = 18.31$, $P < 0.0001$).

Although not statistically significant changes, CAR transfection resulted in a slight (1.2-fold) increase in Luciferin ME metabolism in basal cells which was also reflected in the co-transfected cells (1.4-fold increase).

Effects of CYP1A2 inducers on Luciferin ME metabolism are shown in Figure 6-9.
In untransfected cells, DBA and indirubin each resulted in increased CYP1A2 function (93-fold and 9-fold induction above basal cells respectively).

Transfection of HepG2 cells resulted in a significant reduction of DBA-mediated Luciferin ME metabolism compared to untransfected cells. DBA induction was lower in each of CAR, RXRα ($P < 0.001$) and CAR/RXRα ($P < 0.05$) transfectants.

Significant effects of gene expression on DBA mediated CYP1A2 activity were also observed when transfectants were compared. DBA induction was significantly lower in CAR (24-fold above untransfectected uninduced, $P<0.001$) and CAR/RXRα (27-fold, $P<0.001$) transfected cells than RXRα transfectants (40-fold).
Furthermore, co-transfection of CAR/RXRα resulted in a significant increase in Luciferin ME metabolism above cells transfected with CAR only, ($P<0.05$) following DBA mediated induction.

When transfectants were compared, effects of indirubin mediated induction on CYP1A2 function were also observed. Following CAR transfection, indirubin resulted in a 13-fold increase in CYP1A2 activity above untransfected uninduced cells. This was significantly higher than the induction measured in RXRα transfected cells (7-fold, $P<0.01$).

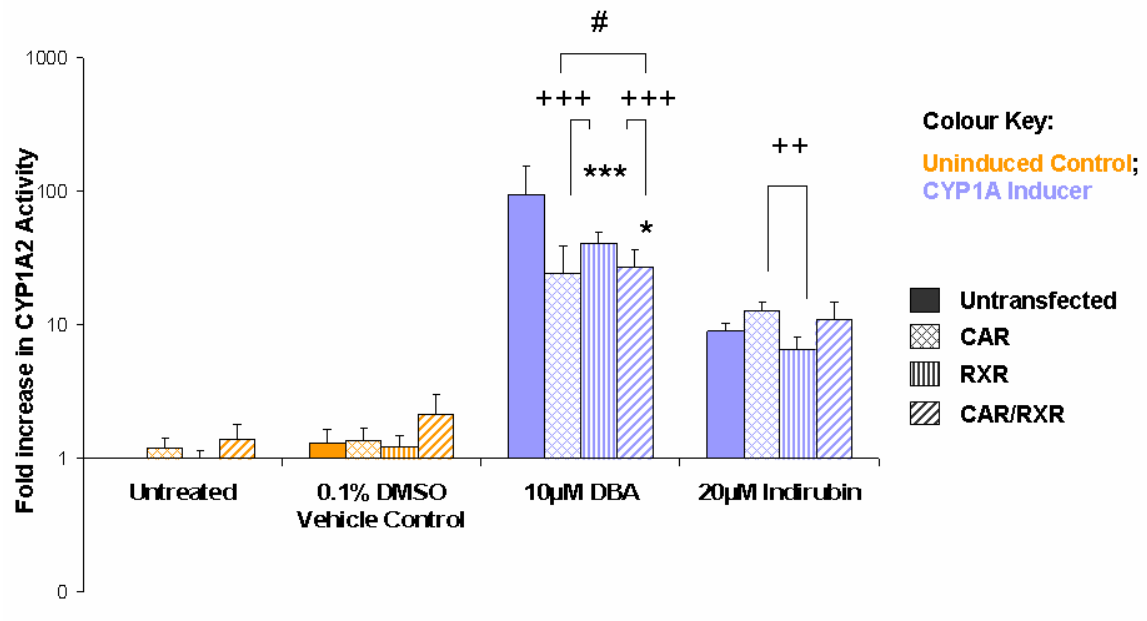


Figure 6-9. Effect of CYP1A2 inducers on Luciferin ME metabolism was measured to assess effect of transfection on CYP1A2 function. CYP1A2 activity was set at 1 for untransfected uninduced cells and compared for mock control and HepG2 cells transfected with CAR, RXRα or CAR/RXRα and treated for 48 hours with inducers. Data is plotted on a logarithmic scale and was analysed by two way ANOVA followed by Bonferroni *post hoc* analysis.

***P<0.05, *** P< 0.01 when transfectants were compared to untransfected uninduced HepG2 cells.**

++P<0.01, +++ P<0.001 when CAR and CAR/RXRα transfectants were compared to RXRα transfected cells.

 # P<0.05 when CAR transfectants were compared to CAR/RXRα transfectants.

As shown in Figure 6-10, CYP3A inducers did not significantly affect CYP1A2 activity.

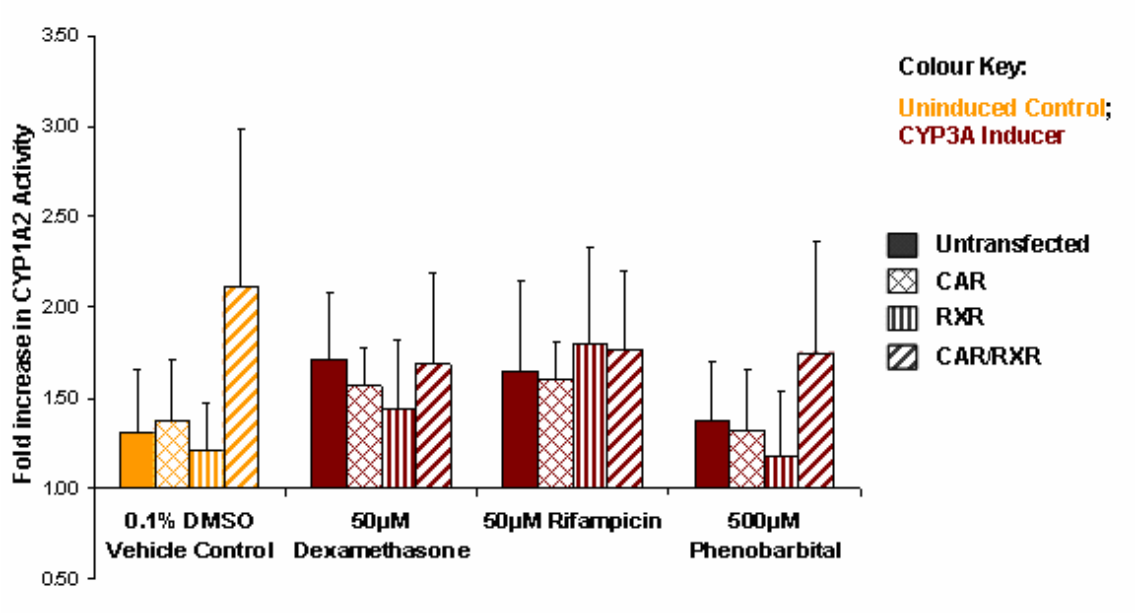


Figure 6-10. CYP1A2 activity was measured in HepG2 cells following transfection with mock control, CAR, RXR*α*, or CAR/RXR*α* and 48 hour treatment with CYP3A inducers. CYP1A2 activity in untransfected uninduced cells was set as 1 and data is expressed as fold increase in activity compared to this.

6.1.2.2.6 Effect of Transient Transfection and Induction on Vivid® BOMFC CYP2B6 Activity

As described in Chapter 3, BOMFC assay was developed in primary human hepatocytes (PHH) to test CYP2B6 metabolism by measuring substrate metabolism in the presence of 100µM clarithromycin. The same method was used to measure CYP function in transfected induced HepG2 cells but was too insensitive in these cells. Although a good standard curve could be generated, and positive readings were obtained with PHH samples (shown in Chapter 3), fluorescence generated with HepG2 samples was no higher than background level.

6.1.2.2.7 Metabolic Viability of Transfected Cells in the Presence of CYP Inducers

WST-1 activity was not significantly different between transfected cells and untransfected cells both with and without addition of inducers (Figure 6-11).

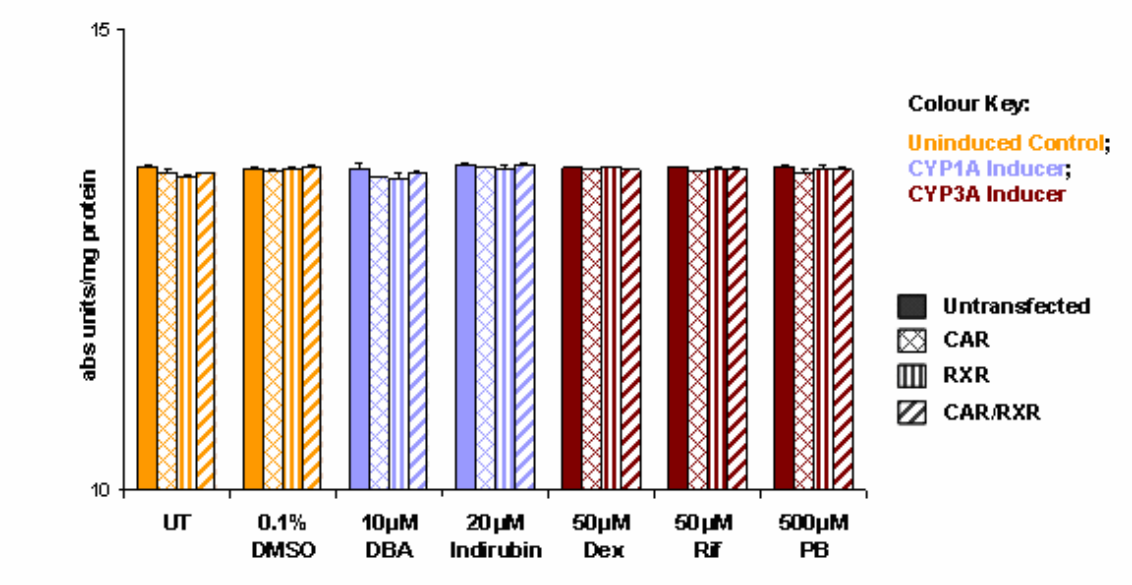


Figure 6-11. WST-1 assay demonstrates that cell viability was unaffected by both inducer treatment and transfection of CAR, RXR*α*, CAR/RXR*α* or mock control.

6.1.2.2.8 A Comparison of PHH and HepG2 Transfectants in Monolayer Culture

CYP3A activity in transfected HepG2 cells treated with 50µM rifampicin was compared with activity measured in freshly isolated PHH (presented in Chapter 3). CYP3A activity in untransfected HepG2 cells was lower than each of the PHH preparations. Rifampicin treatment of CAR and CAR/RXRα HepG2 cells increased CYP3A activity to approach lower levels of activity measured in PHH (Table 6-2).

Table 6-2. CYP3A (Luciferin-BE) activity was higher in freshly isolated primary human hepatocytes than HepG2 cells but the difference was diminished in transfected HepG2 cells treated with rifampicin. Data was compared from PHH isolated from 9 separate donors assayed in quadruplicate, untreated HepG2 cells and HepG2 cells transfected with CAR or CAR/RXRα and treated for 48 hours with inducer. Data is expressed as pmoles d-luc/min/mg protein (mean+/-SD for HepG2 cells, mean, median and range for PHH).

Cell Type	CYP3A Activity (pmoles d-luc/min/mg protein)
PHH Average (9 donors)	0.071
PHH Median (9 donors)	0.051
PHH Range (9 donors)	0.022 - 0.167
Untransfected HepG2 + Rifampicin	0.019 +/-0.002
CAR HepG2 + Rifampicin	0.024 +/-0.002
CAR/RXRα HepG2 + Rifampicin	0.026 +/-0.007

6.1.2.3 Discussion

In this section, Cytochrome P450 induction was improved in HepG2 cells by nuclear receptor transfection and a range of inducers and NR combinations were measured. This was firstly to determine which experimental condition resulted in the highest CYP3A function in order to provide proof of concept for BAL purposes. Secondly NR cross talk was examined by measuring effects of NR transfection on CYP1A2 and CYP3A induction in the presence of a number of inducers of each isoform.

<u>CYP3A Function was Improved Following Nuclear Receptor Transfection</u>

Following transfection of both CAR and CAR with RXRα, CYP3A activity was increased in the absence of inducing agent. This could be due to medium components having an effect either on PXR or CAR expression or CYP3A induction, or CAR exhibiting transcriptional activity in the absence of inducer [274]. For this to be plausible CAR would have to locate to the nuclei of HepG2 cells following transfection, a possibility that is supported by Kowamoto [267] who found that GFP-fused CAR spontaneously localised to the nuclei of transfected HepG2 cells where it could

constitutively activate response elements, resulting in CYP induction. HepG2 cells have been shown to lack expression of the co-chaperone, cytosolic CAR retention protein [275] which, under physiological conditions, retains CAR within the cytoplasm. Lack of this co-chaperone or an increase in HepG2 CAR DNA by transient transfection without concomitant increase in the co-chaperone would result in nuclear accumulation of CAR and so CYP3A induction could occur in the absence of inducing agent.

In the co-transfection experiments, the amount of each DNA used was half the amount added for the single transfectants, which was reflected in the gene expression levels for both CAR and RXRα following co-transfection. Increasing the total amount of DNA added to increase expression to the level measured in single transfectants could result in improved CYP3A induction; however this would be limited by the maximum amount of DNA that can be taken up by the cells.

Other Nuclear Receptors to Regulate CYP Expression

It was demonstrated here that the greatest increase in CYP3A activity following HepG2 CAR and CAR/RXRα transfection was measured following rifampicin induction. Rifampicin mediated CYP3A induction also occurs through binding of PXR to RXRα. Crosstalk between CAR and PXR has been well illustrated *in vivo* through the use of PXR/CAR null mice and *in vitro* evidence confirms that CAR/RXRα heterodimers are able to bind PXR/RXRα response elements in the CYP3A gene [37]. Although PXR expression has been shown to be higher than CAR expression in HepG2 cells [221], it has been extensively demonstrated in a reporter gene system [150, 276] that transfection of PXR into HepG2 cells improves CYP3A induction. Moreover others have shown that transfection of PXR has a stronger effect on CYP3A function than CAR [277]. Co-transfecting PXR with RXRα would equally improve CYP3A activity. However, in previous studies, treatment of human hepatocytes with PXR siRNA was without significant effect on CAR or RXRα and additionally, the expression of CYP1A2 and CYP2D6 mRNA were not affected by the introduction of hPXR-siRNA, which implies that PXR plays no direct functional role in the expression of either of these genes [221].

Manipulation of glucocorticoid receptor (GR) expression in HepG2 cells presents an opportunity to increase expression of PXR, CAR and RXRα [96]. Transfection of GR improves CYP3A function in HepG2 cells and this is further improved by co-

transfection of PXR [278]. Other transcription factors could also act synergistically with CAR and PXR including HNF4α [279].

Effects of Dexamethasone

Lesser effects of dexamethasone in this study may be partly due to the corticosteroid in our culture media raising baseline CYP3A levels making effects of induction difficult to monitor, although it may also be due to an inducer dependent variation in CAR mediated transactivation of CYP which can occur [267]. Dexamethasone mediated CYP3A induction was improved only by co-transfection of CAR and RXRα which suggests that increased expression of both these genes is necessary for CYP3A function to be improved in HepG2 cells by this inducer. Effects of dexamethasone on HepG2 CYP3A function will be explored further in section 6.2.

Transient Nuclear Receptor Transfection Altered AhR Agonist Effects in HepG2 Cells

The effects of CAR and RXRα expression on CYP1A2 function were examined. AhR agonists had differential effects on CYP1A2 activity: Indirubin mediated CYP1A2 function was increased in CAR transfected HepG2 cells and suppressed in RXRα transfected cells. DBA mediated CYP1A2 induction was suppressed by CAR transfection and to a lesser extent by RXRα transfection.

With respect to CYP3A function, indirubin also resulted in an increase in CYP3A activity which was improved following transfection of CAR/RXRα but not in the presence of RXRα alone. DBA resulted in slight (although not statistically significant) induction of CYP3A in all instances.

When these effects are considered together, there are a number of possible implications:

i) AhR ligands such as indirubin (and DBA) can activate CAR resulting CYP3A induction (agonist cross-talk).

ii) AhR ligands bind to CAR and activate CYP1A2 induction (nuclear receptor response element cross-talk).

Both of these possibilities imply cross-talk in mediators of CYP3A and CYP1A2 induction. The slight increase in CYP1A2 function following CAR transfection, both

with and without RXRα, implies that CAR/RXRα is capable of binding CYP1A2 response elements, or controls the regulation of CYP1A by modulating AhR expression. Ueda [280] found a regulatory element in the CYP1A1 gene that simultaneously controlled the induction of both CYP1A1 and CYP1A2 supporting the finding that gene regulation may be controlled by neighbouring genes. In primary human hepatocytes, the PXR agonist rifampicin was shown to increase CYP1A1/1A2 expression [74]. Also it has been demonstrated that treating HepG2 cells with CYP1A2 inducers indigo, indirubin and 3-MC significantly increased CYP3A4 gene expression, whilst the CAR/PXR activator phenobarbital led to an increase in CYP1A1/1A2 expression [136]. Other authors have implied a role for CAR in CYP1A2 induction [271]. Pyrene treatment led to CYP1A2 induction in transgenic $AhR^{-/-}$ mice with a concomitant increase in CAR expression and, in the instance of pyrene treatment, CYP1A2 induction was attributed to CAR activation.

From this, it is tempting to conclude that NR cross-talk is responsible for improved CYP induction following NR transfection; it is plausible, even, that improved CYP3A induction following DBA treatment and CAR transfection is a consequence of increased CAR binding and decreased AhR binding/CYP1A induction by this inducer. Neither of these possibilities, however, fully rationalise the effects of indirubin mediated induction following transfection which leads to a third possibility:

iii) the actions of indirubin as a kinase inhibitor result in improved HepG2 differentiation which in turn results in improved CYP function.

It was shown in Chapter 4 that improved HepG2 differentiation, which arises as a consequence of 3D culture, results in improved CYP function. Indirubin treatment has also been implied in stem cell biology for this purpose [281] and has been shown, in other cell types, to improve differentiation.

Indirubin incubation was shown in HL-60 promyelocytic leukemia cells to act synergistically with VD3 or all-*trans* retinoic acid (ATRA) to improve differentiation following a 72 hour incubation [282]. Neuronal differentiation was also improved after incubation with indirubin and this occurred by inhibition of glycogen synthase kinase 3 β (GSK 3β) and subsequent β-catenin stabilisation [283].

GSK 3β and β-catenin are both involved in the Wnt signalling pathway which controls the expression of cells with predefined fates. GSK 3β regulates levels of the free form of β-catenin. Activation of the Wnt pathway inhibits GSK 3β resulting in cellular accumulation and nuclear translocation of β-catenin whereby it plays a role in gene transcription (Figure 6-12).

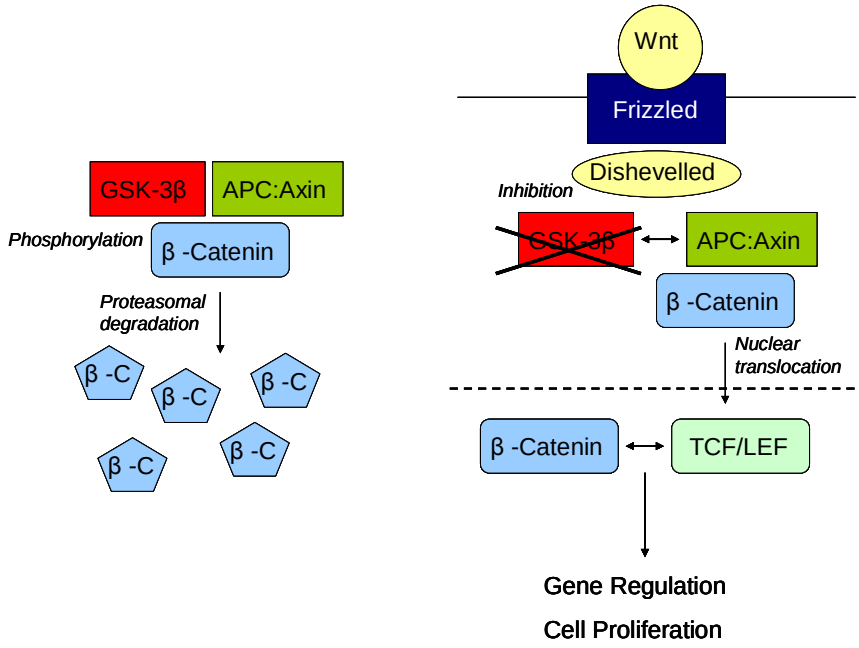


Figure 6-12. β -Catenin is an intracellular signalling molecule whose levels are regulated by proteolytic degradation mediated by GSK-3β, APC-axin complex (left).

In the canonical Wnt pathway (right), Wnt binding of Frizzled receptor results in phosphorylation of cytoplasmic mediator Dishevelled and inhibition of GSK-3. This results in accumulation of β-Catenin which enters the nucleus and interacts with TCF/LEF transcription factors causing gene activation and/or cell proliferation (APC: adenomatous polyposis coli, GSK-3β glycogen synthase kinase 3β, LEF: lymphoid enhancer factor, TCF: T cell factor).

Possible consequences of indirubin exposure or RXRα transfection on β-catenin signalling are shown in Figure 6-13. Indirubin is a GSK-3β inhibitor and as described for the Wnt pathway, indirubin mediated GSK-3β inhibition prevents degradation of β-catenin which is then able to translocate to the cell nucleus and control gene regulation. Crosstalk between RXR and the Wnt/β-catenin pathway has been reported by several authors [284, 285]. RXRα activation has been implied in β-catenin degradation. This effect is not associated with RXRα mediated gene activation, is independent of GSk-3β activity and is thus is not inhibited by GSK-3 inhibitors such as indirubin [286].

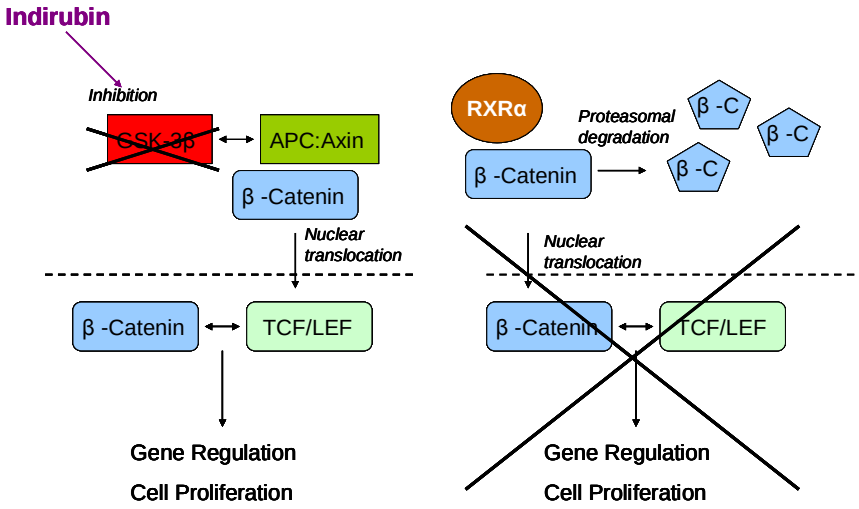


Figure 6-13. Proposed effects of (left) indirubin and (right) RXRα on β-catenin signalling. Indirubin inhibits GSK-3β resulting in cellular accumulation of β-catenin and positive effects on differentiation. RXR-α activation leads to GSK-3β independent β-catenin degradation preventing subsequent nuclear translocation and gene regulation.

Potentially indirubin has an effect on CYP activity which is mediated by increased differentiation. The effect of indirubin mediated GSK-3β inhibition on β-catenin degradation is then lost following RXRα transfection.

In HepG2 cells, nuclear accumulation of mutated β-catenin has been detected which is insensitive to GSK-3β degradation and potentially involved in tumorigenesis [287]. Indirubin exposure results in an accumulation of wild type β-catenin which is involved in cellular differentiation.

Hepatic carcinomas and associated tumour cell lines such as HepG2 have a high nuclear accumulation of phosphorylated RXRα which is insensitive to degradation but lacking in transactivating activity [288]. Accumulation of phosphorylated RXRα has been attributed to MAP-kinase action. Indirubin is also a MAP kinase inhibitor and so could prevent RXRα phosphorylation.

The Wnt signalling pathway, although well characterised is complex and the conclusions described above are based on a number of assumptions which would have to be confirmed experimentally, however the proposed consequences and implications of what is described are summarised below.

a.) Indirubin improves HepG2 differentiation leading to improved CYP activity.

b.) When RXRα is transfected alone it could activate β-catenin degradation pathway, in this instance less is available to act as a co-receptor for CYP induction.

c.) When CAR alone or CAR and RXRα are transfected, CAR binds RXRα resulting in increased CYP transcription; RXRα is then not able to degrade β-catenin.

CYP2B6 Function in HepG2 Cells Following CAR Transfection

Heterodimer formation of CAR with RXRα can result in CYP3A gene induction through activation of XREM and the proximal ER-6 response element, or activation of CYP2B through activation of the phenobarbital-responsive enhancer module (PBREM) [289]. In the experiments performed above (section 6.1.2.2.6), measurement of HepG2 CYP2B6 function using BOMFC metabolism was below the level of quantification even following induction. CITCO is a potent inducer of CYP2B6 which induces HepG2 CYP2B6 mRNA at nanomolar concentrations [74]; a further experiment would be to measure CITCO induction in HepG2 cells. Alternative substrates for CYP2B6 such as buproprion metabolism could also be considered.

CYP3A Function in Transfected HepG2 Cells is Comparable to PHH

When CYP3A function in transfected HepG2 cells was compared with freshly isolated PHH it was found that CYP3A activity in rifampicin induced HepG2 cells transfected with CAR or CAR/RXRα matched CYP3A activity in those PHH with lower levels of activity. Therefore, in a transient system**,** it was demonstrated that increasing HepG2 cell expression of RXRα or CAR results in an increase in CYP3A function, whereby inducible CYP function is significantly improved relative to untreated cells to levels similar to those measured in hepatocytes from donors with lower CYP metabolising capacity. In a stable expression system this method could be used to provide CYP3A function within a BAL. In order to confirm this, CYP3A function was explored in cells stably transfected with CAR and maintained in 3D culture.

6.1.3 Creation of a Stable Cell Line for Encapsulation

HepG2 cells were transfected with pTracer CAR construct using Lipofectamine™ 2000 reagent as described above. To create stable transfectants the cells were placed under

selective pressure using blasticidin S-HCL. Cells under selective pressure were expanded in culture and then encapsulated and maintained in 3D culture to measure CYP function in cells cultured in this format.

6.1.3.1 Methods

6.1.3.1.1 Establishing a Blasticidin Sensitivity in HepG2 Cells

A 10mg/ml solution of blasticidin S-HCL (Invitrogen 461120) was prepared in sterile water. This solution was filter sterilised and dispensed into single-use aliquots which were stored at -20°C until required. Medium containing blasticidin (Bsd) was stored at +4°C for no longer than one week. To determine Bsd sensitivity in HepG2 cells, $2.5x10^5$ cells were added to 6 well plates in αMEM complete and allowed to attach overnight. Cells were incubated with 0, 2, 4, 6, 8 or 10 µg/ml Bsd for up to 2 weeks. Selection medium was replenished every 48 hours to remove dead cells that had accumulated in the medium and cells were passaged as required. From this experiment it was determined that 4-6µg/ml was a sufficient concentration of Bsd to prevent cell growth after 2 weeks of culture.

6.1.3.1.2 Stable Transfection of HepG2 Cells

HepG2 cells were transfected in 12 well plates with 1.6µg of CAR using a reverse transfection method, as described in section 6.1.2.1. The following day, transfection medium was replaced with αMEM complete containing 10% FCS. Cells were then detached from the plate using a cell scraper, syringed 3x with a 21g needle and transferred to a new well of a 12-well plate. The following day, culture medium was replaced with antibiotic selection medium comprising αMEM complete (10% FCS) with 6µg/ml Bsd. Medium changes were performed every 48 hours.

After 2 weeks those cells that had not been killed by the antibiotic had begun to proliferate. These cells were passaged by trypsinisation into a 6 well plate and were cultured in this format for one week after which time they were transferred into a T25 flask. Cells were passaged once and then, in order to remove those cells with lower gene expression, Bsd concentration was increased to 10µg/ml. Once cell proliferation recovered, cells were maintained at this level of selection and were expanded in culture until a confluent T175 was attained (a portion of cells was frozen at each passage using the protocol described in Chapter 2); this was achieved in 6 passages for CAR and at this point cells were used for encapsulation. On the same day as encapsulation, cells

were plated for monolayer assays to confirm gene expression and CYP3A function in CAR transfectants independent of 3D culture.

6.1.3.1.3 CYP3A Induction in Stable Transfectants: Monolayer Culture

CAR transfectant or control HepG2 cells (untransfected) were added to 96 well plates ($2.5x10^6$ cells/well) in 100µM αMEM complete 10% FCS (+/- 10µg/ml Bsd) and allowed to attach overnight. Cells were then treated for 48 hours with 50µM rifampicin at which point CYP3A activity was measured by Luciferin BE assay. Total protein content was assessed by the BCA assay. For the CAR transfectant, Luciferin BE activity was also measured in the presence of 1-100µM clarithromycin or vehicle (0.1% MeOH) control.

6.1.3.1.4 Gene Expression in Stable Transfectants: Monolayer Culture

RNA samples were isolated from control (untransfected) HepG2 cells or CAR transfectants maintained in monolayer culture and treated for 48 hours with 50µM rifampicin. Gene expression was performed by qRT-PCR as described in Chapter 2.

6.1.3.1.5 CYP3A Induction in Encapsulated Transfectants

HepG2 CAR transfectant cells were encapsulated using the Inotech method described in Chapter 2. Beads were resuspended at a ratio of 0.25ml beads: 8ml HG medium and maintained in static culture, in the presence of 10µg/ml Bsd, for 8 days. Medium was replenished every other day and viability and cell growth assessed by phase contrast microscopy and FDA/PI staining.

At day 6 post encapsulation, 0.1% DMSO vehicle or 50µM rifampicin were added to the growth medium. Following a 48 hour induction period, CYP function was measured by Luciferase assay or testosterone metabolism. Total protein content was assessed by the BCA assay.

6.1.3.2 Results

6.1.3.2.1 HepG2 CAR Expression Following Blasticidin Selection

Selection of Bsd resistant (CAR expressing) HepG2 cells was detected by monitoring GFP expression using fluorescence microscopy (Figure 6-14).

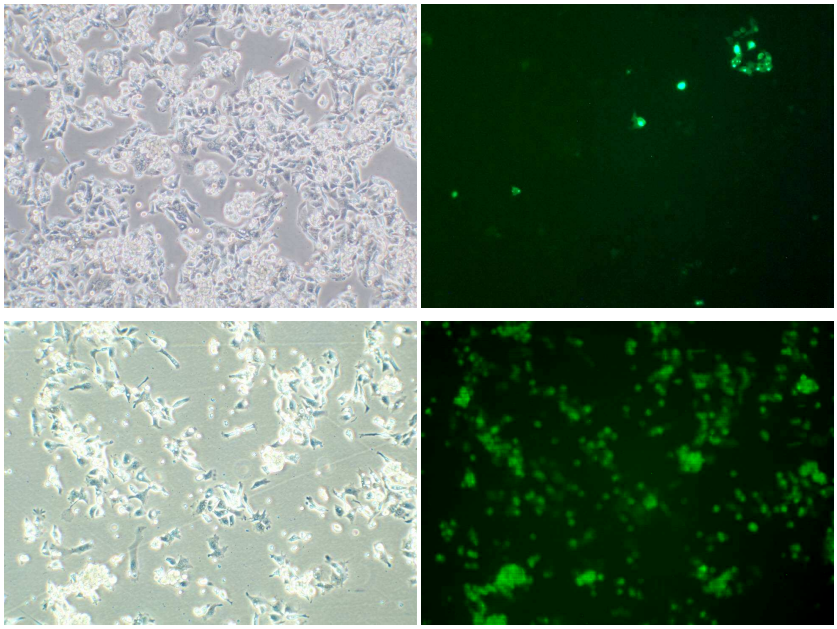


Figure 6-14. (left) Phase contrast and (right) GFP expression in HepG2 cells which have been transfected with pTracer construct and undergone blasticidin selection. The top images show HepG2 transfectants after 3 weeks selection in 6µg/ml Bsd. Selection was subsequently increased to eliminate cells with lower gene expression. Bottom images were recorded following 3 passages in 10µg/ml Bsd. Images are x10 magnification.

6.1.3.2.2 Gene Expression in Monolayer CAR Transfectant

Gene expression was compared in mRNA isolated from CAR transfectant (following Bsd selection) and control (untransfected) HepG2 cells, following 48 hour treatment with 50µM rifampicin in monolayer culture. Increased expression of CAR was confirmed in the transfected cells that had undergone Bsd selection. There was also increased CYP expression in the CAR transfectant. The greatest increase observed was in CYP3A4 mRNA (1240 fold), followed by CYP3A7 (150 fold); there was less of a difference in CYP1A2 expression (7 fold) between the two cell types (Figure 6-15).

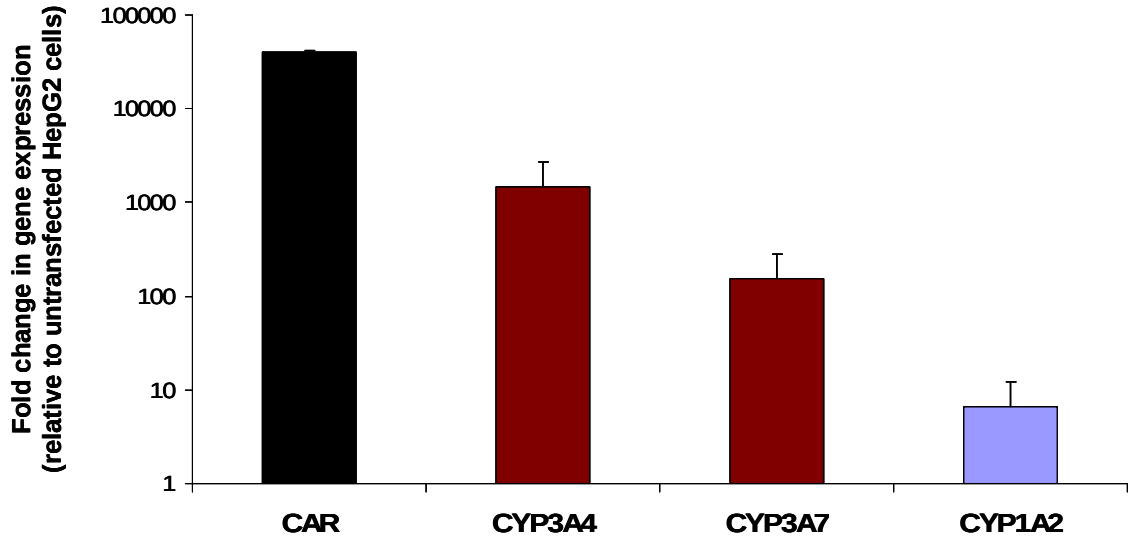


Figure 6-15. Gene expression was compared in control (untransfected) HepG2 cells and Bsd selected CAR transfectant treated in monolayer culture for 48 hours with 50µM rifampicin. For each gene, expression was normalised to ribosomal 18s expression and change in expression then calculated relative to untransfected HepG2 cells. This was repeated twice and data is presented as n=2 +/- range.

6.1.3.2.3 CYP3A (Luciferin BE) Function in Monolayer CAR Transfectant

CYP3A (Luciferin BE) activity was compared in CAR transfectant (following Bsd selection) and control (untransfected) HepG2 cells, following 48 hour treatment with 50µM rifampicin in monolayer culture. There was slight (but not significant) increase in Luciferin BE activity in the CAR transfectant (1.2 fold relative to untransfected cells) (Figure 6-16, top). Luciferin BE activity was significantly diminished (F (4, 0.0004)

=11.92, P< 0.01), in a dose-dependent manner, when measured in the CAR transfectant in presence of the CYP3A inhibitor clarithromycin (Figure 6-16, bottom).

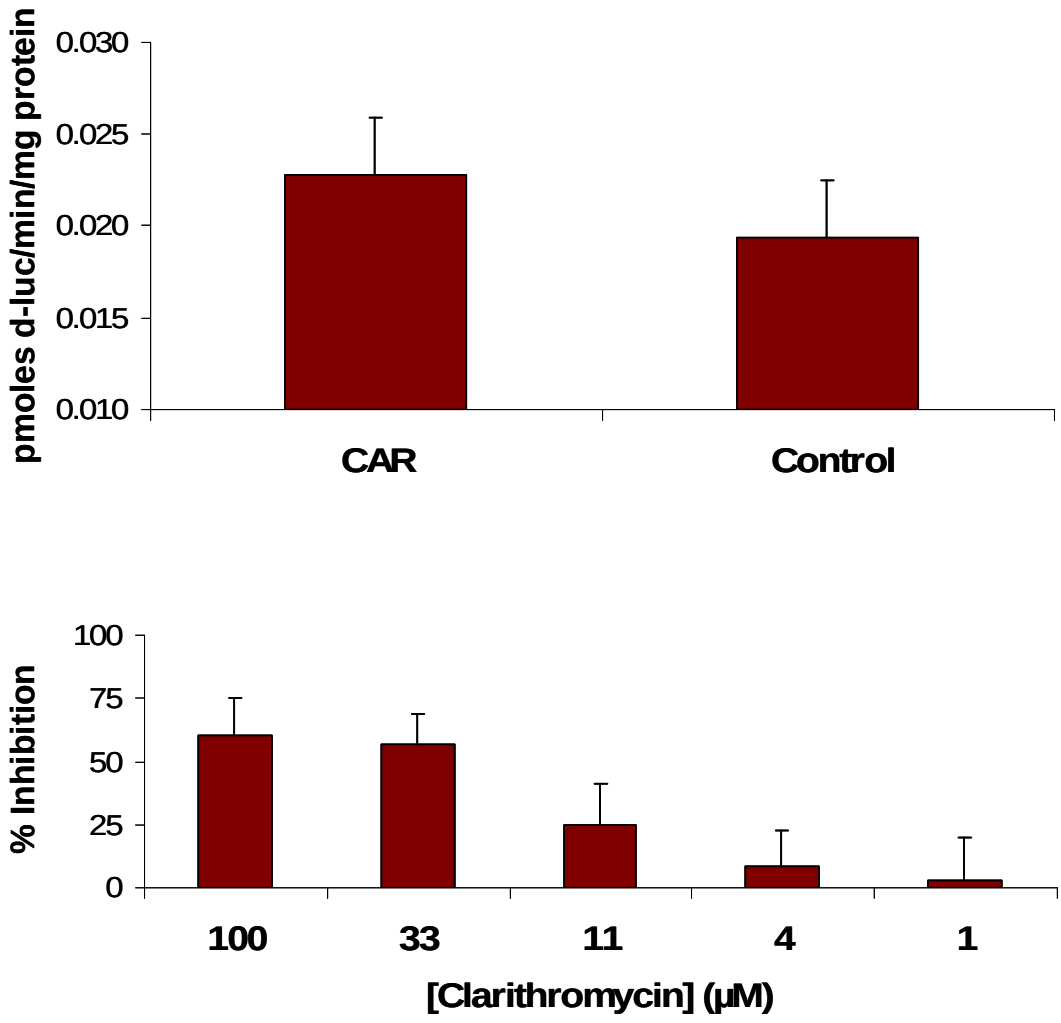


Figure 6-16. CYP3A (Luciferin BE) activity was measured in CAR transfectants that had undergone blasticidin selection prior to encapsulation. CYP3A activity was higher in CAR transfected HepG2 cells than in untransfected control following 48 hour treatment with 50µM rifampicin (top) and was inhibited when measured in the presence of clarithromycin in HepG2 CAR transfectant (bottom). Data is presented as mean (n=3)+/-sd and is expressed as (top) pmoles d-luc/min/mg of protein or (bottom) % inhibition of CYP3A activity relative to cells without inhibitor.

6.1.3.2.4 3D Culture of Transfectants Maintained in Static Culture

Figure 6-17 shows the growth and viability of encapsulated transfectants maintained in static culture. FDA (live cell) staining demonstrates proliferation and spheroid formation throughout 8 days of culture. PI (dead cell) staining indicates that at day 8, when functional assays were performed, viability was high.

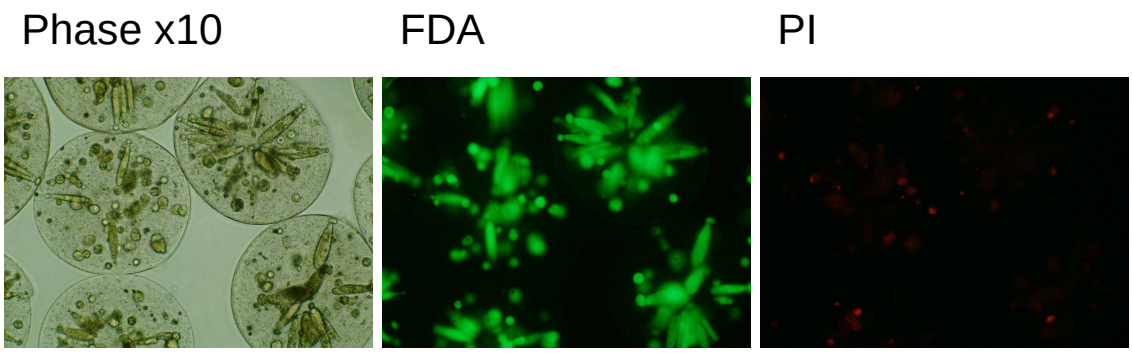


Figure 6-17. Cell viability staining (x10 magnification) of encapsulated HepG2 cell CAR transfectant following 8 days of static culture.

6.1.3.2.5 CYP3A Function (Luciferase Activity) in Encapsulated CAR Transfectant

CYP3A (Luciferin BE) activity was compared in CAR transfectant following Bsd selection or control (untransfected) HepG2 cells that had been cultured in 3D format for 8 days and treated for 48 hours (from day 6) with 50µM rifampicin or 0.1% DMSO vehicle. Improved induction was observed in the CAR transfectant.

Under basal conditions Luciferin BE metabolism was slightly higher in the CAR transfectant (1.2 fold above control cells), and was further increased (2.6 fold) following rifampicin mediated induction, although neither of these changes were statistically significant (Figure 6-18, top).

CYP3A activity was also measured using the alternative (and more selective) CYP3A substrate Luciferin PFBE and in this assay there was a greater effect of CAR expression on CYP3A induction (Figure 6-18, bottom). Data was analysed by two-way ANOVA. There was a significant effect of CAR expression on Luciferin PFBE activity ($F(1, 0.008)=94.99$, $P < 0.001$) and a significant effect of rifampicin induction in these cultures ($F(1, 0.005)=54.87$, $P < 0.001$). There was also a significant interaction

between the two factors (F (1, 0.004)=52.88, P <0.001); rifampicin mediated induction was increased in the CAR transfectant. Under basal conditions, Luciferin PFBE was higher in the CAR transfectant (2.8 fold above control cells) and was further increased following rifampicin exposure (21 fold above control cells). In both assays, extent of rifampicin mediated CYP3A induction was therefore greater in the CAR transfectant.

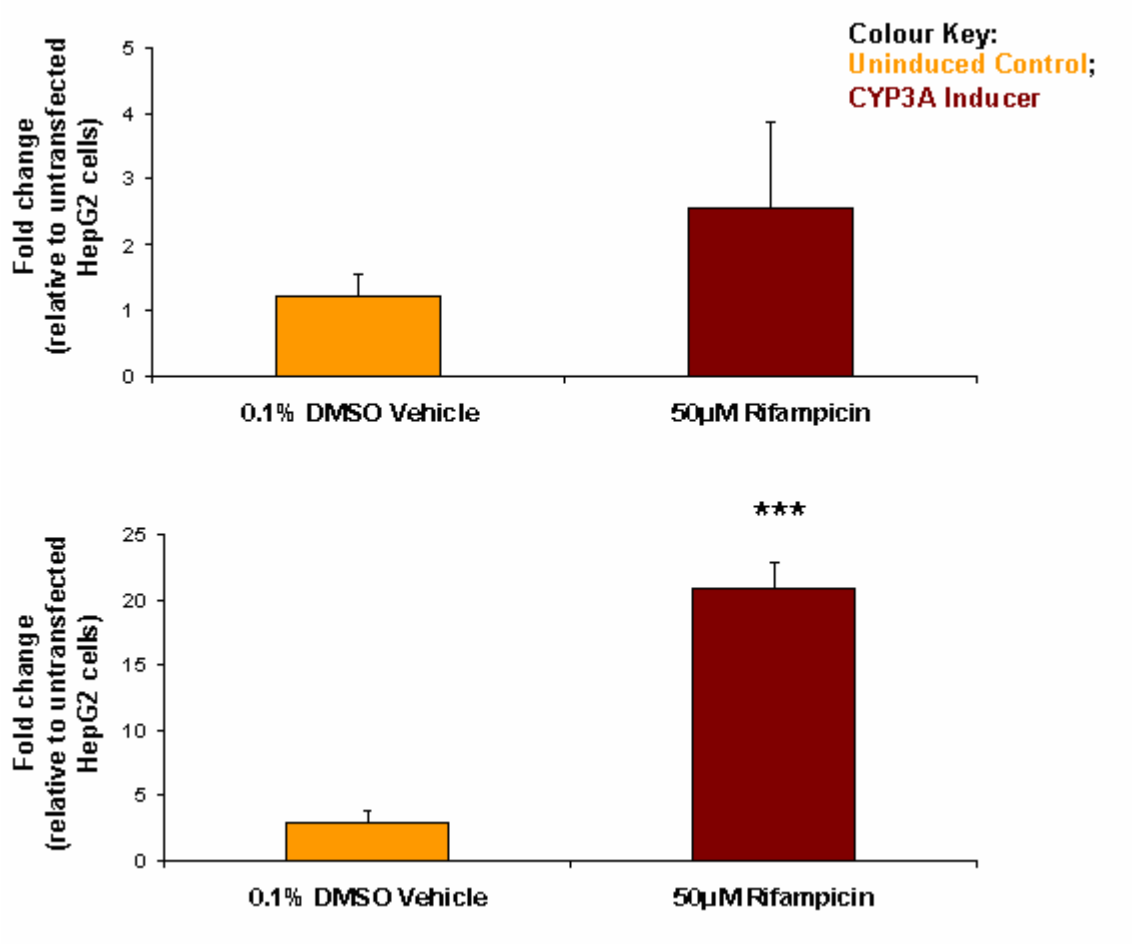


Figure 6-18. CYP3A induction was compared in control (untransfected) HepG2 cells and Bsd selected CAR transfectant maintained for 8 days 3D format and treated for 48 hours (on day 6) with 50µM rifampicin or 0.1% DMSO Vehicle. CYP3A activity was measured using (top) Luciferin BE or (bottom) Luciferin PFBE substrates. For each assay fold change in CYP activity of the CAR transfectant was calculated and is expressed here as mean (n=4)+/- sd. Data analysis was by two-way ANOVA and Bonferroni post test. ***P < 0.001 when compared to untransfected HepG2 cells.

6.1.3.2.6 Testosterone Metabolism in Encapsulated CAR Transfectant

Substrate metabolism was analysed in Bsd selected CAR transfectants that had been treated for 48 hours with 50µM rifampicin and incubated for up to 8 hours with 200µM testosterone (TST). Included in the same analysis run was supernatant from control

(untransfected) HepG2 cells treated in the same manner, and freshly isolated PHH treated for 1 hour with 200µM TST.

Both CAR transfectant and control (untransfected) HepG2 cells were able to metabolise TST; the retention characteristics of metabolites produced by CAR transfectants and freshly isolated PHH were broadly similar and differed from untransfected HepG2 cells (Figure 6-19).

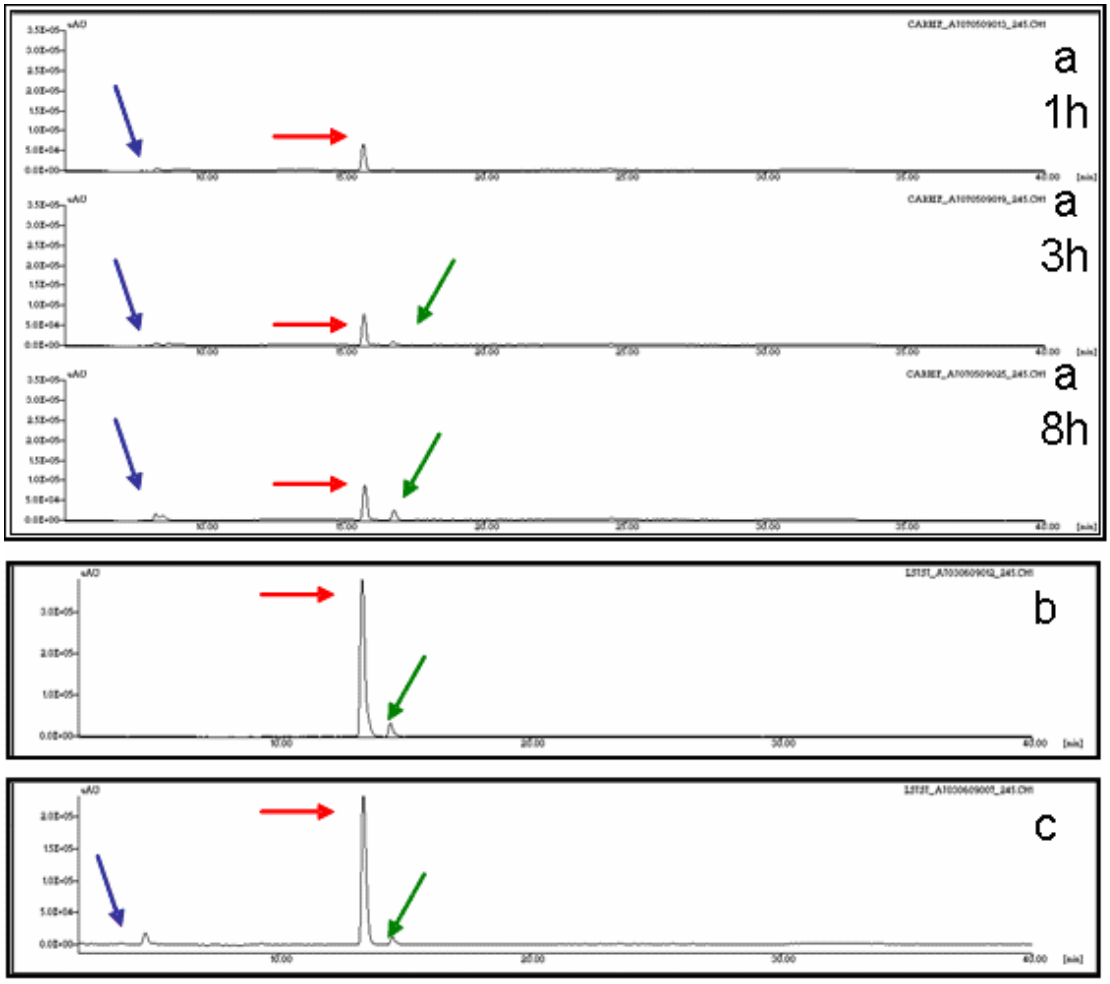


Figure 6-19. HPLC was used to measure metabolism of testosterone in (a) CAR transfectants treated for 48 hours with 50µM rifampicin in 3D culture and then incubated with 200µM TST for 1, 3 or 8 hours, (b) control (untransfected) HepG2 cells treated in with 50µM rifampicin in 3D culture and then incubated with 200µM TST for 8 hours or (c) freshly isolated PHH incubated with TST for 1 hour. TST spike is indicated by →. TST metabolites are indicated by →/→. One representative trace is shown of 4 independent measurements.

6.1.3.3 Discussion

In 3D culture, HepG2 cells need to be proliferated for extended periods (around 8 days) to maximise the benefits of spheroid formation. This presents a limitation when attempting to measure effects mediated by gene expression in a transient system; furthermore large numbers of cells are required for encapsulation which makes a transient transfection approach impractical. HepG2 cells stably transfected with CAR were thus proliferated under Bsd selection to allow encapsulation.

Prior to encapsulation, increased CAR expression was confirmed in cells under Bsd selection in monolayer culture. CAR transfectants also has increased CYP3A expression following rifampicin exposure and CYP3A function was improved in these cells: this function was diminished by the CYP3A inhibitor clarithromycin.

CYP1A2 expression was also higher in the CAR transfectant following rifampicin induction. Others have also demonstrated a CYP1A2 induction following rifampicin induction both in PHH and HC-04 cells [74, 160] which adds further credence to the hypothesis that multiple isoforms of CYP can be induced by the presence of a single transfectant.

In 3D culture, basal activity was improved in the CAR transfected cells relative to untransfected cells. Moreover the increase in CYP3A function detected in the CAR transfectant was greater following rifampicin treatment. This implies that upregulation in CYP3A induction following 3D culture, which (as demonstrated in Chapter 4) is limited in HepG2 cells, can be achieved when CAR expression is increased.

CYP3A induction of CAR transfected and control (untransfected) HepG2 cells in 3D culture was compared using Luciferin BE and Luciferin PFBE assays and there were differences between the two substrates. As described in Chapter 1, Luciferin BE is also metabolised by CYP4F12 and the CYP3A affinities of each substrate are different: Luciferin BE is metabolised by CYP3A7 > CYP3A4 > CYP3A5, Luciferin PFBE is metabolised by CYP3A5>CYP3A7>CYP3A4; the difference in affinities between individual isoforms is less for Luciferin PFBE. In Chapter 4, it was demonstrated that CYP3A induction achieved with either substrate in untransfected HepG2 cells was equivalent in monolayer culture. Following 3D culture of the CAR transfectant, greater upregulation of function was measured using the Luciferin PFBE substrate.

Gene expression data derived from monolayer cells, together with the TST metabolism profile of the encapsulated CAR transfectant, implies that improving CAR expression results in upregulation of CYP3A4 function; CYP3A4 expression was increased to a greater extent than CYP3A7 expression in the CAR transfectant following rifampicin treatment. Others have demonstrated that CAR binds the CYP3A4 promoter with greater affinity than the CYP3A7 promoter [46, 84]. Potentially this was reflected in the results obtained here and demonstrated at a functional level in 3D culture following rifampicin induction.

Creation of a single transfectant potentially could create an imbalance between HepG2 expression of CAR and of its obligate co-receptor RXRα, hence limiting CYP induction [67, 94]. Here this was demonstrated in a transient system (section 6.1.2); co-transfection of CAR and RXRα increased the extent of CYP3A function that could be induced in HepG2 cells. Using the approach described in this Chapter, stable expression of more than one gene would have required the use of more than one selection antibiotic. This would have placed additional stress on the transfected cells, with a resultant impact on proliferation and viability and so would need to be addressed in order for a dual gene transfer method to be applicable within a BAL.

6.2 Cytochrome P450 Function in HepG2 Cells from an Alternative Source (BALG2 Cells)

In was hypothesised that previous adaptation to the supplemented αMEM used within the Liver Group is responsible for some regulation of the CYP function described throughout this thesis. Further to this, others have noted differences in HepG2 cell function depending upon their original source [51]. In order to confirm this, a new HepG2 stock was obtained from the HPACC. For clarity these cells will be referred to as BALG2. These cells were either maintained in a simple culture medium (DMEM) or adapted to αMEM complete. CYP induction was then compared in these cells and in LG HepG2 cells.

6.2.1 Methods

6.2.1.1 Adaptation to Culture Media

BALG2 cells were maintained in DMEM (as per delivery medium) as described in Chapter 2 for intestinal cell culture. After cells had been passaged at least once post thawing and reached 50% confluency, growth medium was switched to complete

culture medium as described for HepG2 culture (Chapter 2). Cells were maintained in this medium for at least 3 passages prior to any further studies.

HepG2 cells were maintained in αMEM as described in Chapter 2. After cells had been passaged at least once post thawing and reached 50% confluency, growth medium was switched to DMEM as described for intestinal cell culture (Chapter 2). Cells were maintained in this medium for at least 3 passages prior to any further studies.

6.2.1.2 Cytochrome P450 Induction in BALG2 Cells

HepG2 cells were induced as in Chapter 4 and CYP function measured by testosterone or Luciferase assay. Luciferin ME assay was performed in the presence of 10µM sulfaphenazole.

6.2.1.3 Gene Expression in BALG2 Cells

HepG2 or BALG2 cells were plated in 12 well plates ($2.5x10^5$ cells/well) in 1ml of either DMEM or αMEM complete and allowed to attach overnight. The following day, 50µM dexamethasone was added. Cells were incubated with inducer for 48 hours. RNA was then isolated and gene expression determined by qRT-PCR using the methods described in Chapter 2.

6.2.2 Results

Cytochrome P450 activity was compared in HepG2 or BALG2 cells adapted to either DMEM or αMEM and then treated for 48 hours in monolayer culture with inducers. Data was analysed by two-way ANOVA and Bonferroni post test.

6.2.2.1 A Comparison of CYP1A2 (Luciferin ME) Activity in HepG2 and BALG2 Cells

There was a significant effect of both cell type ($F(3, 1.4x10^{-7}) = 3.96$, $P < 0.01$) and inducer ($F(6, 1.4x10^{-6}) = 19.03$, $P < 0.0001$) on CYP1A2 activity, however there was no significant interaction between the two effects.

The following results are shown in Figure 6-20.

Under basal conditions, CYP1A2 activity was slightly higher in BALG2 αMEM cells (1.3 fold). Basal activities of HepG2 and BALG2 DMEM cultures were comparable.

The effects of DBA (10µM) and rifampicin (50µM) on CYP1A2 activity were unaffected by cell type or media composition.
Indirubin had a greater effect on substrate metabolism in cells cultured in DMEM; CYP1A2 activity was higher than HepG2 αMEM for both HepG2 DMEM (1.5 fold, $P<0.05$) and BALG2 DMEM (1.5 fold).

There were no additional increases observed in CYP1A2 function following dexamethasone (50µm) and phenobarbital (500µM) exposure in HepG2 DMEM and BALG2αMEM. However following treatment with both these inducers, CYP1A2 activity was improved in BALG2 DMEM to levels matching BALG2 αMEM.

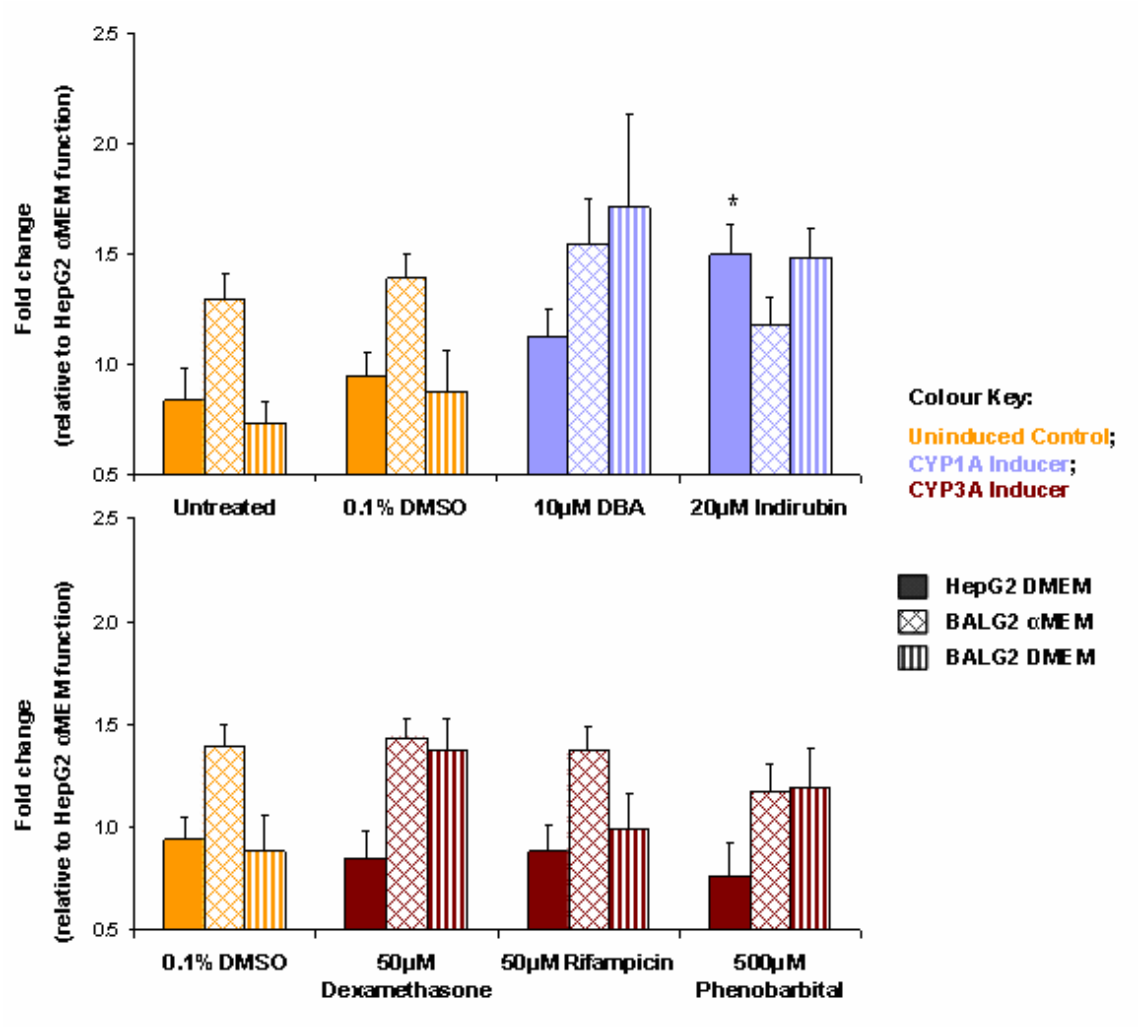


Figure 6-20. CYP1A2 (Luciferin ME) activity was measured in HepG2 cells or BALG2 cells cultured in αMEM or DMEM and treated for 48 hours with (top) CYP1A inducers and (bottom) CYP3A inducers. Data is expressed as fold change in activity compared to corresponding induction in HepG2 cells cultured in αMEM. Data analysis was by two-way ANOVA and Bonferroni post test (n=6 +/-sd). * P<0.05 when compared to HepG2 cells in αMEM.

6.2.2.2 A Comparison of CYP3A (Luciferin BE) Activity in HepG2 and BALG2 Cells

There was a significant effect of both cell type ($F(3, 0.003)=48.61$, $P< 0.0001$) and inducer ($F(6, 0.001)=10.34$, $P< 0.0001$) on CYP3A function, however no significant interaction between the two variables.

The following results are shown in Figure 6-21.

Under basal conditions, CYP3A activity was higher in BAL G2 cells cultured in both αMEM (1.6 fold higher than HepG2 αMEM) and DMEM (1.4 fold).

The effects of rifampicin (50µM) and phenobarbital (500µM) on CYP3A activity were unaffected by cell type or media composition. Activity was significantly higher than observed in HepG2 cells in BALG2 αMEM and BALG2 DMEM treated with rifampicin (1.5 fold $P<0.001$ and 1.5 fold $P<0.001$) and phenobarbital (1.5 fold $P<0.01$ and 1.6 fold $P<0.001$), however the increase in activity observed following treatment with inducers was comparable between the cell types.

Dexamethasone mediated CYP3A induction was affected by both cell type and media composition. Substrate metabolism following dexamethasone exposure (50µM) was higher in BALG2 αMEM (2.0 fold above HepG2αMEM, $P<0.001$) and further improved in BALG2 DMEM (2.7 fold above HepG2 αMEM, 2.5 fold above HepG2 DMEM, $P<0.001$).

The effects of DBA (10µM) and indirubin (20µM) on CYP3A activity were unaffected by cell type or media composition.

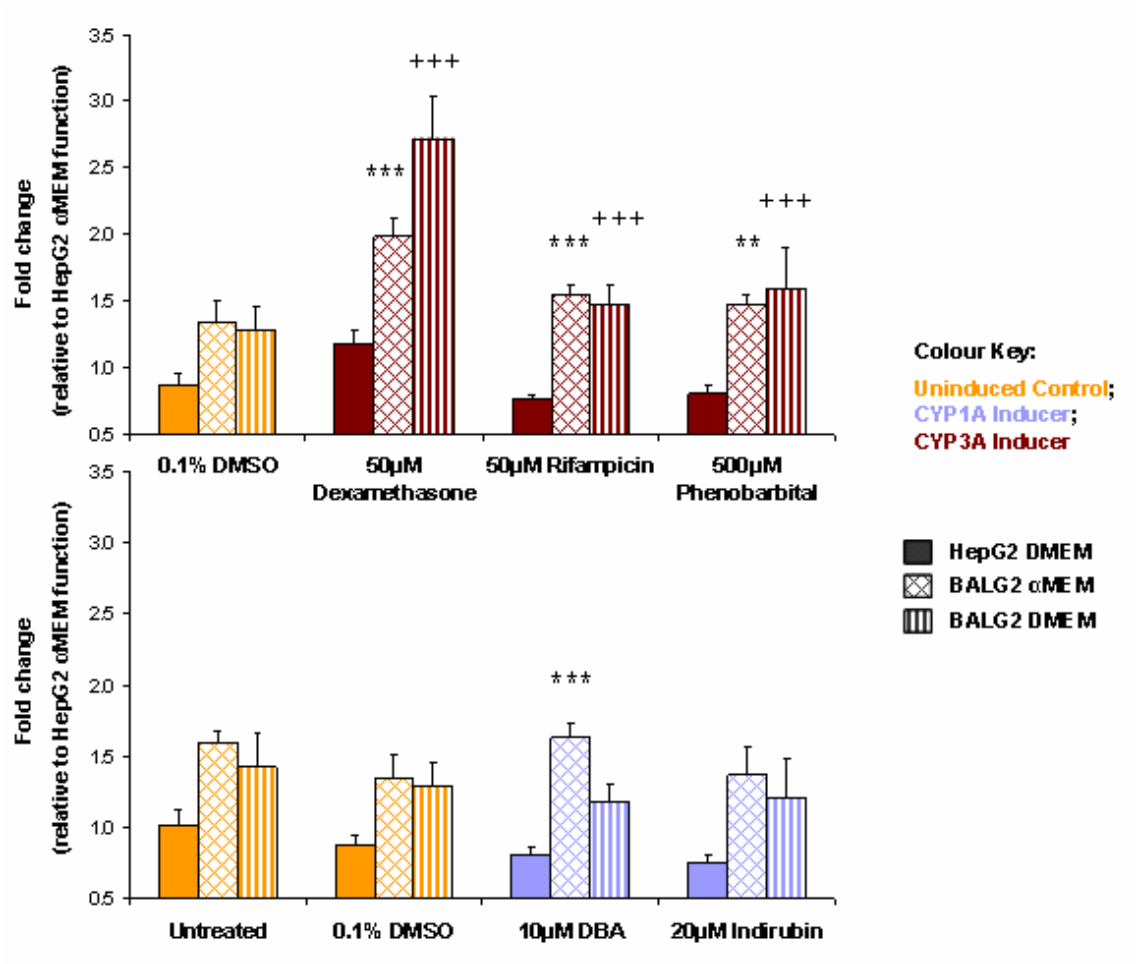


Figure 6-21. CYP3A (Luciferin BE) activity was measured in HepG2 cells or BALG2 cells cultured in αMEM or DMEM and treated for 48 hours with (top) CYP1A inducers and (bottom) CYP3A inducers. Data is expressed as fold change in activity compared to corresponding induction in HepG2 cells cultured in αMEM. Data analysis was by two-way ANOVA and Bonferroni post test (n=6 +/-sd). ** P<0.01, P<0.001 when compared to HepG2 cells in αMEM. +++ P<0.001 when compared to HepG2 cells in DMEM.

6.2.2.3 A Comparison of CYP mRNA Expression in BALG2 and HepG2 Cells

CYP expression was measured in HepG2 or BAL G2 adapted to either αMEM or DMEM and treated for 48 hours with 50µM dexamethasone and was dependent on both cell type and media composition (Figure 6-22).

CYP3A4 expression was higher in BALG2 cells. For both HepG2 and BALG2, CYP3A4 expression was comparable in cells cultured in either αMEM or DMEM.

CYP3A7 expression was higher in BALG2 cells. For both HepG2 and BALG2, CYP3A7 expression was increased in cells cultured in αMEM.

CYP1A2 expression was higher in BALG2 cells although the difference was less apparent than for CYP3A isoforms. For BALG2, CYP1A2 expression was higher in cells cultured in αMEM.

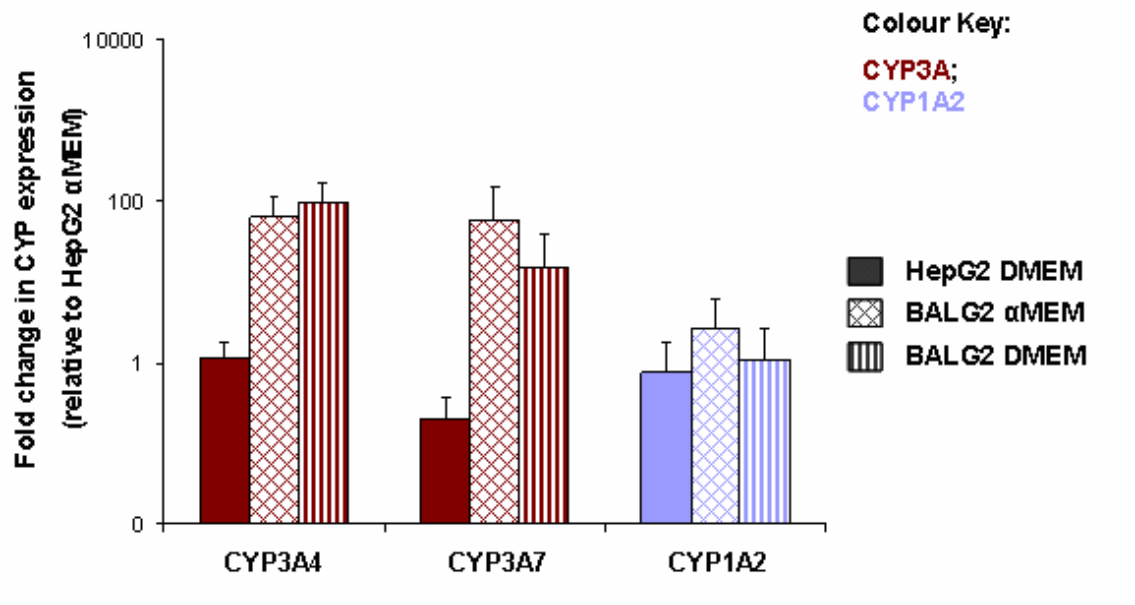


Figure 6-22. CYP expression in HepG2 cultured in DMEM and BALG2 cultured in αMEM or DMEM was compared to expression in HepG2 cells cultured in αMEM (normal LG conditions). In each instance CYP expression was normalised to ribosomal 18s expression and change in expression then calculated relative to normal LG conditions. Data is presented as n=2 +/- range.

6.2.2.4 A Comparison of Nuclear Receptor mRNA Expression in BALG2 and HepG2 Cells

NR Expression was compared in HepG2 and BALG2 cells adapted to either αMEM or DMEM and then treated for 48 hours with 50µM dexamethasone, and was dependent on both cell type and media composition (Figure 6-23).

CAR expression was lower in BALG2 cells. For both HepG2 and BALG2, CAR expression was improved in cells cultured in αMEM.

PXR expression was comparable between the two cell types. For HepG2 cells, expression of PXR was slightly higher in cells adapted to DMEM.

RXRα expression was doubled in BALG2 cells cultured in αMEM compared to HepG2 cells under the same conditions. For both cell lines RXRα expression was higher in cells cultured in DMEM.

GR expression on HepG2 cells was comparable between media types. GR expression was lower in BALG2 cells cultured in αMEM but was increased to levels matching HepG2 cells in BALG2 cells cultured in DMEM.

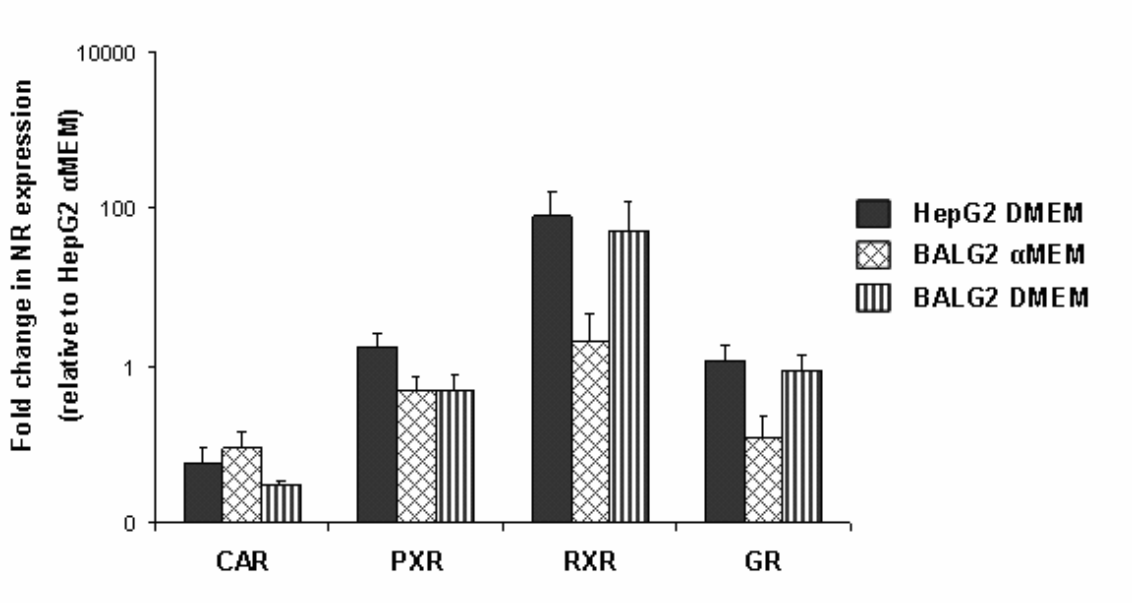


Figure 6-23. NR expression in HepG2 cultured in DMEM and BALG2 cultured in αMEM or DMEM was compared to expression in HepG2 cells cultured in αMEM (normal LG conditions). In each instance, cells were treated for 48 hours with 50μM dexamethasone. NR expression was normalised to ribosomal 18s expression and change in expression then calculated relative to normal LG conditions. Data is presented as n=2 +/- range.

6.2.2.5 Testosterone Metabolism in BALG2 Cells

Monolayer HepG2 or BALG2 cells were cultured in αMEM complete in the presence of 50µM rifampicin for 48 hours. Cells were then exposed to 200µM testosterone for 8 hours. Included in the same analysis run was supernatant from freshly isolated PHH incubated for 1 hour with 200µM testosterone. Testosterone metabolism profiles were comparable for HepG2 and BAL G2 cells however neither cells produced 6β-OHTST as a product of testosterone metabolism (Figure 6-24).

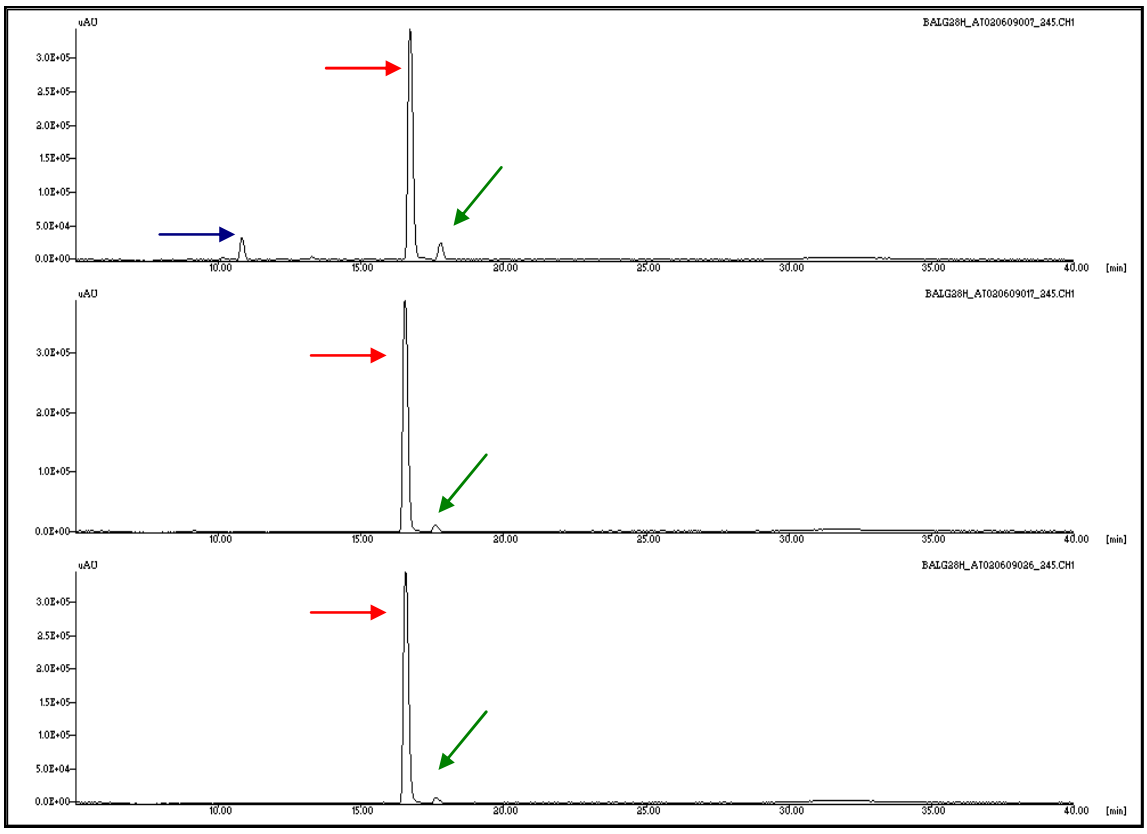


Figure 6-24. HPLC was used to measure metabolism of testosterone in (middle) HepG2 cells and (bottom) BALG2 cells treated with 50µM rifampicin in monolayer culture and then incubated with 200µM TST for 8 hours, or (top) freshly isolated PHH incubated with 200µM TST for 1 hour. TST spike is indicated by →. TST metabolites are indicated by →/→. One representative trace is shown of 4 independent measurements.

6.2.2.6 A Comparison of PHH and BALG2 in Monolayer Culture

CYP3A activity in HepG2 or BALG2 cells treated with 50µM rifampicin or dexamethasone was compared with activity measured in freshly isolated PHH (presented in Chapter 3). CYP3A activity in HepG2 cells was lower than each of the

PHH preparations. Rifampicin or dexamethasone treatment of BALG2 cells increased CYP3A activity to approach lower levels of activity measured in PHH (Table 6-3).

Table 6-3. CYP3A (Luciferin-BE) activity was higher in freshly isolated primary human hepatocytes than HepG2 cells but not BALG2 cells. Data was compared from PHH isolated from 9 separate donors assayed in quadruplicate and monolayer HepG2 (cultured in αMEM) or BALG2 (cultured in αMEM or DMEM) cells treated for 48 hours with 50μM dexamethasone or rifampicin. Data is expressed as pmoles d-luc/min/mg protein (mean+/-SD for HepG2 cells, mean, median and range for PHH).

Cell Type	CYP3A Activity (pmoles d-luc/min/mg protein)	
PHH Average (9 donors)	0.071	
PHH Median (9 donors)	0.051	
PHH Range (9 donors)	0.022 - 0.167	
	+ DEX	**+ RIF**
HepG2 αMEM	0.014 +/-0.001	0.019 +/-0.002
BALG2 αMEM	0.029 +/-0.004	0.029 0.004
BALG2 DMEM	0.039 +/-0.014	0.028 +/- 0.008

6.2.3 Discussion

Hewitt and Hewitt [51] emphasise the need for independent confirmation of CYP expression in HepG2 cells and propose that the belief these cells are lacking in drug metabolising capacity is not entirely accurate. An evaluation of two sources of HepG2 cells carried out by these authors demonstrated that both Phase I and II enzyme metabolising capacities depend on source and culture conditions. Doostdar *et al.* [137] suggest that reported absence of CYP activity in HepG2 cells is (partly) due to the use of inappropriate culture media and other authors have established that media components and supplements including serum and growth factors and dexamethasone, in addition to oxygenation of cultures can all affect HepG2 cell CYP function [5, 55, 228].

Overall CYP1A2 activity was better in BALG2 cells cultured in αMEM. CYP3A function and mRNA expression in BAL G2 cells was substantially higher than in HepG2 cells in both media types; both cell types were able to metabolise testosterone but neither produced 6β-OHTST. The higher CYP capacity of BALG2 cells could be due to a strain difference between the HepG2 cells as well as a consequence of the multiple passages and media types that the HepG2 cells had previously been subject to [51, 55, 139].

Glucocorticoid agonists, such as dexamethasone, prednisolone, or hydrocortisone, increase human CAR expression and upregulate PXR and CYP3A4 expression in HepG2 cells [55], and induce RXRα at nanomolar concentrations in primary human hepatocytes [95]. Here, the effect of induction particularly for dexamethasone was greater in cells cultured in DMEM.

The effect of glucocorticoids in culture medium was also examined by measuring NR expression in cells cultured in DMEM or αMEM complete following treatment with dexamethasone. Both cell types cells cultured in DMEM had higher expression of RXRα and GR following dexamethasone treatment. Potentially components of the αMEM such as hydrocortisone or insulin maintain a more physiological environment for the cells which has a regulatory effect on NR expression [203, 205].

Activity in BALG2 cells, described above, was measured in monolayer culture and following induction fell within the range of levels measured for PHH. As described in Chapter 4, it was found that 3D culture resulted in a 3-fold increase in CYP3A activity in HepG2 cells. If the same could be achieved in BALG2 cells then their CYP3A activity would achieve the average level measured in PHH. Since the cells used in this study had been maintained in culture for a maximum of 8 passages, monitoring the impact of longer term adaption to αMEM on CYP function in BALG2 cells would be critical.

6.3 Conclusions

In a transient system, increased CAR expression improves CYP3A function in HepG2 cells. Using an appropriate stable expression system, genetic manipulation of nuclear receptor expression could improve CYP function within a HepG2 BAL .However, as described above, this approach is limited. In hepatocytes, multiple NR and transcription factors are involved in the induction of CYP enzymes. Whilst increasing NR by genetic manipulation improves CYP3A function it does not account for the full range of NR and transcription factors that can be achieved in a well differentiated cell line.

With regard to HepG2 cells, CYP function varied markedly between cell sources and it was demonstrated here, in agreement with others, that HepG2 function is media dependant and strain dependent; BALG2 cells appear to provide better function and in some instances were able to match PHH levels of function. This emphasises the need to maintain a well characterised cell bank to provide a BAL cell source.

CHAPTER 7 General Discussion

A Summary of Findings and Future Work

The overall aim of this thesis was to identify a cell source that could provide xenobiotic metabolism within the LG BAL. Specifically, CYP1A2 and CYP3A function were considered.

7.1 Characterising and Improving CYP Function in the LG BAL

The first aspect of this thesis was to define how much Cytochrome P450 function a BAL would need to provide by measuring activity in adult human liver. Secondly, the limitations of the existing LG BAL, comprising alginate-encapsulated HepG2 cells, with regard to Cytochrome P450 function were investigated. Lastly, methods to improve CYP3A function within the LG BAL were explored.

7.1.1 How Much Function is Sufficient for a BAL?

Primary human hepatocytes (PHH) are the closest *in vitro* model to human liver and although Cytochrome P450 function is affected by culture conditions, PHH produce the metabolic profile of a drug most similar to that found *in vivo*. Freshly isolated PHH were therefore used to define the level of CYP activity required of a BAL. For both CYP1A2 and CYP3A there was a large donor dependent variation in CYP activity, confirmed by measuring function in human liver microsomes (HLM) isolated from the same donor; this range of function was used to define the limits within which CYP function in other cell lines should fall.

One caveat to these limits is that although PHH were isolated from normal liver tissue, these samples were not obtained from healthy individuals; all samples were obtained following tumour resection as a result of secondary colon cancer, and so are not representative of an entirely normal population [290]. Although cells in a BAL will supplement the patient's residual function, due to the diverse aetiology of acute liver failure (ALF), and the wide range of factors that affect CYP function *in vivo,* a target population is complicated by definition. Others have shown that PHH isolated from tissue derived from therapeutic hepatectomy have a lower metabolic competency compared to samples obtained from elective biopsy (up to 50% decrease in the case of CYP3A4 function, although this data was derived 24 hours post isolation) [291]. This

suggests that aiming to achieve a level of function within the lower range measured here in PHH may be insufficient; a value at least twice the lowest level of function in PHH would be a more realistic aim.

7.1.2 What Were the Limitations of HepG2 Cells?

CYP activity could be induced in HepG2 cells, moreover, 3D culture resulted in significant upregulation of CYP1A and CYP3A function, which was further improved by microgravity culture. However, although HepG2 cells were able to metabolise testosterone, they did not perform the CYP3A4 mediated 6β-hydroxylation reaction. CYP3A7 capacity could provide substrate metabolism in most cases and although following RCCS culture CYP3A function fell within the limits measured in PHH, it was still limited in HepG2 cells.

Within the LG BAL, FFP will be used in place of FCS to avoid the use of animal derived products. HepG2 cell proliferation is improved in the presence of FFP and synthetic capacity is retained (unpublished data). In Chapter 3, it was shown that ECOD activity decreased less in PHH maintained in 10% FFP than those maintained in 10% FCS, which also infers a role for FFP in maintaining differentiation.

In Chapter 4, the impact of FFP exposure on CYP function in HepG2 cells was considered. It was demonstrated that exposure to FPP improved ECOD activity and CYP1A2 function, but there was a limiting effect of FFP exposure on CYP3A function, which reduced the restricted level of function that could be achieved in HepG2 cells. This effect will have to be accounted for in any BAL relevant cell line. Exposure to FFP did not however diminish rifampicin mediated CYP3A induction therefore the activity lost as a result of FFP exposure could be partially restored. Moreover the improvement in viable cell density within a bead cultured with FFP would offset the decreases in CYP activity.

Unfortunately, when HepG2 cells were maintained in 3D culture, although CYP3A function was improved, the effect of rifampicin induction was lost. This suggested that the extent of CYP3A function that could be obtained in HepG2 cells was limited in some way, and also, that mechanisms that governing CYP expression were not

improved in 3D culture. Additional methods to provide BAL CYP3A function were therefore explored.

7.1.3 Why Are Intestinal Cells Unsuitable?

In Chapter 5, intestinal cells were evaluated for their BAL potential. Promisingly, both Caco2 cells and LS147T cells adapted well to 3D culture, with a differentiating effect of this culture format on CYP3A function, which was upregulated in both cell lines. However, of the two cell lines considered, neither could be used in the LG BAL. Caco2 cells were unable to provide sufficient CYP3A function even in 3D culture. LS147T cells had far higher CYP3A function, which matched freshly isolated PHH, and following induction were able to perform 6β-hydroxylation of testosterone in 3D culture. However, viability of LS147T cells was low in the encapsulated cells and, since this can probably be attributed to their debris shedding properties, it is unlikely that this could be fully resolved through optimisation of culture conditions. Within a BAL, a DNA binding membrane is included to prevent transfer to the patient, but these membranes have a limited capacity, therefore any BAL component that adds to their burden should be carefully justified. Given that that LS147T cells are of intestinal origin, their role within a BAL would be limited, hence, in spite of the CYP3A function that can be achieved in encapsulated LS147T cells, their use within a BAL would be too great a compromise.

7.1.4 Do HC-04 Cells Have LG BAL Potential?

In Chapter 5, CYP3A function under BAL conditions of the immortalised human liver cell line HC-04 was also evaluated. In monolayer culture under equivalent conditions, HC-04 and HepG2 CYP3A functions were similar. Luciferin BE activity was lower than PHH and was comparable for both cells lines.

Alginate encapsulated HC-04 cells were successfully cultured over a prolonged period of time. Expression of CYP1A2, 3A4 and 3A7, as well as nuclear receptors that govern their expression, were all upregulated following alginate encapsulation. Unlike HepG2 cells, CYP3A induction was improved in 3D culture. Following rifampicin induction, Luciferin BE activity in d8 HC-04 spheroids matched the mean value measured in freshly isolated PHH, although, similarly to HepG2 cells, HC-04 cells did not produce detectable quantities of the CYP3A4 testosterone metabolite 6β-OHTST, and mRNA expression data indicates that CYP3A7 is a greater contributor to HC-04 CYP3A function than CYP3A4.

The rate of HC-04 cell proliferation in 3D culture was decreased relative to HepG2 cells, even when cells were encapsulated at double the seeding density and prolonged culture resulted in a decrease in viable cell number. This can partly be attributed to decreased oxygenation and nutrient availability and a build up of cytotoxic waste products, and could therefore be improved using RCCS culture [230].

In future work, proliferation of HC-04 cells could be optimised by culture in the presence of FFP. Possibly, (based upon HepG2 data) this would be at the cost of some CYP3A function. Reduced CYP3A function following FFP exposure could be partially restored by rifampicin induction, and so would be balanced against the benefits of improved proliferation, viability and therefore per bead performance that could be achieved by culturing HC-04 cells, in the presence of FFP, in microgravity culture.

Due to regulatory concerns associated with HC-04 cells, including traceability and cell line contamination (both by ECM of bovine origin and adventitious microbial elements), it was decided that these use of these cells within the LG BAL would be inappropriate. In the future, an alternative cell line could be developed in a similar manner to HC-04 cells for BAL use.

7.1.5 Can CYP Function be Improved in HepG2 Cells?

In Chapter 6, methods to improve HepG2 CYP3A function were considered. It was demonstrated in a transient system that increasing CAR and RXRα expression resulted in improved CYP3A function. In 3D culture, CYP3A induction was improved using a CAR transfectant and these cells had an improved testosterone metabolism profile.

In 3D culture, the effect of CAR transfection only was examined. The balance of nuclear receptor (NR) expression that can be achieved through this approach should be considered, since the amount of genes that can be altered by genetic manipulation in one system is a limiting factor, and altering one gene alone may be insufficient. This is apparent for HNF4α, which is implicated in CYP expression, yet the comparatively high levels of HNF4α (relative to PHH) that have been found in HepG2 cells are alone insufficient to promote CYP expression; this suggests further elements such as PXR and CAR are required [45, 279, 292].

Nuclear receptors share ligands, dimerisation partners, response elements and target genes [82], and within this thesis, as summarised in Figure 7.1, inducers were selected that would reflect this.

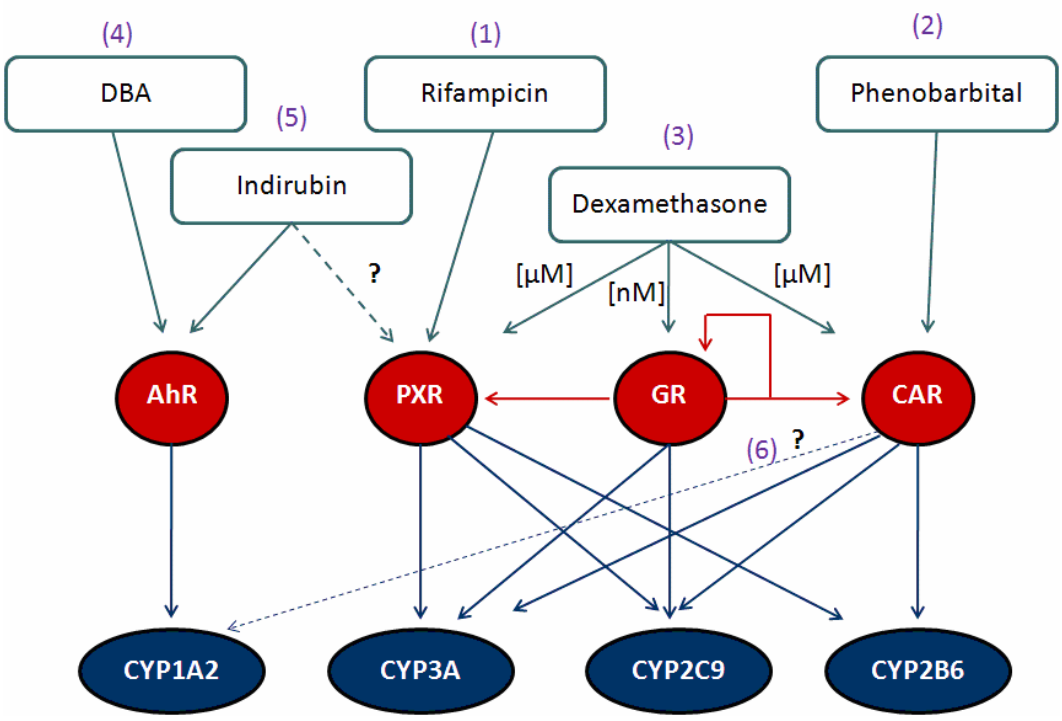


Figure 7-1. Pathways of CYP induction by chemical inducers and instances of receptor cross-talk discussed in this thesis: (1) Rifampicin binds PXR leading to the induction of multiple CYP isoforms. (2) Phenobarbital has an indirect influence on the CAR receptor leading to induction of multiple CYP isoforms. (3) Physiological concentrations of glucocorticoids (GC) bind at GR to increase PXR, CAR and GR levels; high concentrations of GC directly activate PXR/CAR causing induction of multiple CYP isoforms; GR activation also directly influences CYP expression. (4) DBA binds AhR resulting in CYP1A2 induction. (5) In chapters 4 and 6 it was proposed that in addition to acting as an AhR agonist, indirubin acts at other nuclear receptors (probably PXR) resulting in induction of multiple CYP isoforms. (6) In Chapter 6, CAR transfection led to an increase in CYP1A2 activity.

Transfection of PXR or CAR both increase CYP3A function in HepG2 cells. Co-transfection of PXR and CAR would probably be of limited benefit since they share the same response elements and would compete for their shared co-receptor RXRα. It was demonstrated in Chapter 6 that transfection of RXRα alone has a limiting effect on CYP induction, and RXRα ligands have been shown to decrease CYP3A4 activity in human cell lines [188]; however, co-expression of CAR or PXR with RXRα in a stable cell line could lead to increased expression of multiple CYP isoforms.

Within HepG2 cells, sustained expression of multiple genes could be achieved by using a multicistronic vector with liver specific promoters to reduce promoter down-regulation by the host cells. Potentially, more than one cell type would have to be developed to account for the interaction of transcription factors involved in CYP regulation. CYP2D6 in particular should be considered since it metabolises a relatively large but distinct set of substrates (up to 25% of hepatic xenobiotic metabolism) but is not chemically induced. Others have shown that re-expression of CYP2D6 can occur following functional expression of C/EBPα in HepG2 cells [148]. Transcription factors, including C/EBP, may not be upregulated by HepG2 cells in 3D culture, but act synergistically with PXR and CAR to regulate CYP expression [229].

A multiple gene transfer approach could also be used to directly express multiple CYP isoforms and a similar approach has recently been described in HepG2 cells using adenovirus vectors to express multiple CYPs [293]. Equally, there would be considerable limitations to the number of CYP isoforms that can be expressed at a functional level. Furthermore, transfection of CYP enzymes alone limits the opportunity for their expression to be manipulated in culture.

7.2 Further Considerations

7.2.1 Indirubin Improves Cytochrome P450 Function by Multiple Mechanisms

Indirubin is an AhR agonist and is established as a CYP1A inducer. In this thesis it was shown that indirubin exposure also resulted in increased CYP3A function in HepG2 and LS147T cells but not HC-04 cells. In Chapter 6, a potential mechanism was proposed whereby rather than directly inducing CYP, indirubin acts as a mediator of differentiation leading to improved CYP activity.

A similar role was proposed for VD3 in intestinal cell lines in Chapter 5 (and by others), moreover, a combined effect of VD3 treatment and 3D culture on CYP3A function in LS147T cells was observed. Additionally, cellular differentiation and VD3 mediated CYP3A induction can be augmented by the retinoic acid receptor ligand ATRA [188, 282]. Synergistic effects of RXRα signaling and indirubin in the mediation of cellular differentiation through regulation of the Wnt/β-catenin pathway were also described in Chapter 6.

Indirubin could therefore have a role in HepG2 cells as both an inducer of CYP function and also a promoter of hepatocyte differentiation, and both processes could be altered by mediators of CYP induction such as RXRα. This could be explored further by measuring the effects of indirubin on other hepatocyte specific functions/markers of differentiation, including albumin and alpha-1 antitrypsin synthesis. Indirubin could also improve PXR signalling through repression of protein kinase C [294].

The cell line specific effects of indirubin on CYP3A function infers not only that the effect is greatest in less differentiated cells such as carcinoma derived cell lines, but also that HC-04 cells are better differentiated in monolayer culture and hence, that differentiating effects of indirubin were abrogated. Additionally, the effects of indirubin on CYP3A function were diminished in HepG2 cells in the presence of FFP. This could limit the role of indirubin in the better differentiated 3D culture environment that is provided by the LG BAL.

7.2.2 What is Preventing CYP Expression/Protein Translation and is this Recovered Following Improved Differentiation?

DNA methylation is a mechanism by which hepatoma cells regulate expression of genes deemed unnecessary to promote survival/proliferation, and has been implicated as a restricting factor in expression of xenobiotic metabolising enzymes in HepG2 cells [295, 296]. Gene expression can be regained by exposing cells to a demethylating agent such as 5-aza-2'-deoxycytidine. However, for the long term culture of cells on a large scale, and for cells to be used in a BAL, this is not a suitable approach. There is a risk of patient exposure to 5-aza-2'-deoxycytidine, which is toxic and would also be detrimental to the cells over a prolonged exposure period. It is also probable that the genes will be re-silenced when the demethylating agent is removed.

Dannenberg *et al.* [295] also found that exposure of HepG2 cells to a demethylating agent resulted in the down-regulation of C/EBPβ, which is responsible for the transcription of CYP enzymes as well as additional hepatocyte specific genes. The C/EBPβ gene can form two isoforms from a single mRNA: liver activation protein (LAP) which is the full-length isoform; and liver inhibitory protein (LIP), which is a truncated isoform that lacks most of the transactivation domain and serves as a dominant negative regulator of LAP transcriptional activity [295-297].

C/EBPβ LAP/LIP ratio has a strong influence on CYP3A4 expression/induction and also modulates flavin-containing monooxygenase 3 (FMO3) expression. A low LAP/LIP ratio is associated with foetal phenotype and is also found in HepG2 cells maintained in monolayer culture [45, 295-297]. In HepG2 cells, transfection of recombinant LAP or LIP causes corresponding increase or decrease in rifampicin mediated CYP3A4 activation. Although the expression of C/EBPβ has been measured in 3D cultures of HepG2 cells [229], the LAP/LIP ratio needs to be defined and, if deficient, could be manipulated in HepG2 cell to improve CYP3A function.

7.2.3 Maintaining CYP Expression Following Induction (Post Translational Stabilisation)

As discussed in Chapter 1, CYP activity *in vivo* takes time to return to basal levels following induction. The same would have to be true of HepG2 cells if chemical induction were to be applicable to provide BAL CYP activity. In addition to the data presented in previous chapters, ECOD activity was also measured in DBA treated HepG2 cells up to 48 hours post induction and was maintained above basal levels in the absence of inducer (this data is presented in Appendix B).

Post translational stabilisation of CYP enzymes could also be improved by the presence of catalase, which provides antioxidant activity and protects CYP enzymes from drug induced degradation (including acetaminophen) [298] and proteasome inhibitors, which would decrease enzyme turnover as well as NR degradation [82, 299]. When considering the development of a system that can be cryopreserved, both of these methods could potentially stabilise CYP expression in the period between bioreactor thawing and patient exposure; addition of catalase is part of our cryopreservation protocol.

7.2.4 Considering Flavin-Containing Monooxygenases

Non-CYP mediated oxidative reactions can also play a major role in xenobiotic metabolism, and FMOs catalyse NADPH-dependent monoxygenation of a number of xenobiotics. FMO3 is the predominant form of the FMO family expressed in human liver that is involved in this process, and could therefore provide an alternative drug metabolism pathway. Although it is generally accepted that CYP enzymes have the more significant role in xenobiotic metabolism, there is a degree of substrate overlap

between CYPs and FMO3- examples include clozapine, ketoconazole, cimetidine, tamoxifen and agrichemicals [15, 16].

FMO3 has not been reported at functional levels in HepG2 cells [296]. Possibly, it is upregulated by 3D culture or could be replaced by genetic manipulation. Supplementation of transcription factors that control CYP metabolism, including HNF4α , but not C/EBPβ or HNF3β, could also augment FMO3 expression in HepG2 cells [296], alternatively FMO3 could be directly transfected (into HepG2 cells). This approach should be simpler than attempting to restore the complex CYP enzyme system and has the additional advantage that relatively few compounds inhibit FMO3 compared to CYP enzymes. By decreasing the dependence on CYP mediated catalysis and distributing drug metabolism across multiple enzyme systems, FMO3 could thus provide oxidative drug metabolism function that is additive to both the patient's own liver and the CYP function provided by HepG2 cells. In practice, this may not be so straightforward since FMO3 metabolites may inhibit the CYP system [15]. In order to measure the success of this approach, it would be necessary to distinguish between FMO3 and CYP mediated catalysis in HepG2 cells. *In vitro* the two systems differ; FMO catalysis typically occurs faster, and experimental conditions in a microsomal system (such as increased pH and presence of detergents) could be manipulated to favour FMO activity. FMO enzymes are, however, more thermally labile than CYPs; this is especially evident in HLM systems and so a suitable assay to monitor gene expression would also be required.

7.3 What the Current System Can Achieve

When considering an established, well characterised, cell line that is readily available, HepG2 cells remain an appealing candidate for the LG BAL. HepG2 cells have substantial synthetic capacity, can proliferate to form a sufficient biomass and although in monolayer culture their CYP function can be low, this can be improved, in particular by 3D culture.

Within this thesis, Cytochrome P450 function was considered with respect to CYP1A2 and CYP3A enzymes. To obtain a broader representation of drug metabolism, further work would include measuring the metabolism profiles of a number of drug substrates

that are metabolised by additional CYP isoforms; Phase II metabolism should also be confirmed.

Strain dependent differences in function, that were profiled in Chapter 6, further demonstrate that HepG2 cell function can be highly variable, dependent on a number of factors. BALG2 cells had a greater basal level of CYP function and higher CYP3A expression, yet the extent of CYP induction that was achieved in BALG2 cells in monolayer culture was, in most cases, no greater than in the LG HepG2 cells. Although this demonstrates that CYP3A induction may still be limited, by a combination of chemical induction and genetic manipulation, BALG2 cells have the potential to provide a range of Cytochrome P450 functions that in 3D culture are equivalent to that measured in human liver.

Appendix A

Adaption of HC-04 Cells to LG Culture

Initially, HC-04 cells were maintained in HCM media, supplemented as described in Chapter 2, on a matrix of collagen and fibronectin. It was therefore necessary to determine both their ability to proliferate and their CYP function when cultured with or without culture matrix in either HCM or αMEM complete.

Methods

Preparation of Collagen Coated Plasticware

1mg/ml Fibronectin
1mg/ml Collagen Solution from Calf Skin Type I
10% Bovine Serum Albumin (BSA)
HBM (Lonza)
T25 Primaria Flasks (BD)
96 Well Plates
A solution containing 0.01mg/ml fibronectin, 0.03mg/ml collagen solution and 0.01mg/ml BSA was prepared in HBM. 1.5ml or 100µl of this solution was added to T25 flasks or 96 well plates respectively which were incubated for 2 hours at 37°C. Excess liquid was then removed prior to addition of cells.

Adapting Culture Conditions of HC-04 Cells

Cells were maintained in HCM media on collagen/fibronectin matrix in T25 flasks. At ~ 60% confluency, HCM media was replaced with αMEM complete. Cells were maintained for one passage in αMEM complete, and at the subsequent trypsinisation were then plated in T25 flasks without matrix.

CYP Induction in Monolayer Culture

CYP induction was measured in both HC-04 cells that had been adapted to their culture conditions and cells that had been cultured in HCM on matrix prior to CYP induction as described in Chapter 5. HepG2 cells were cultured in the same conditions as a comparator. This experiment was performed weekly over a period of 1 month to determine whether activity was maintained during time in culture and effect of prolonged adaption to alternative culture conditions.

Results

CYP3A Activity Comparison: Original versus Adapted Conditions

CYP3A activity following rifampicin induction was measured in HC-04 cells cultured in HCM on collagen matrix or in αMEM on tissue culture plastic (Figure A-1). CYP3A activity was higher in the αMEM adapted cells, however, under these conditions the effect of rifampicin induction was lost. Activity was comparable but not better than HepG2 activity and was less than the lowest level measured in freshly isolated PHH.

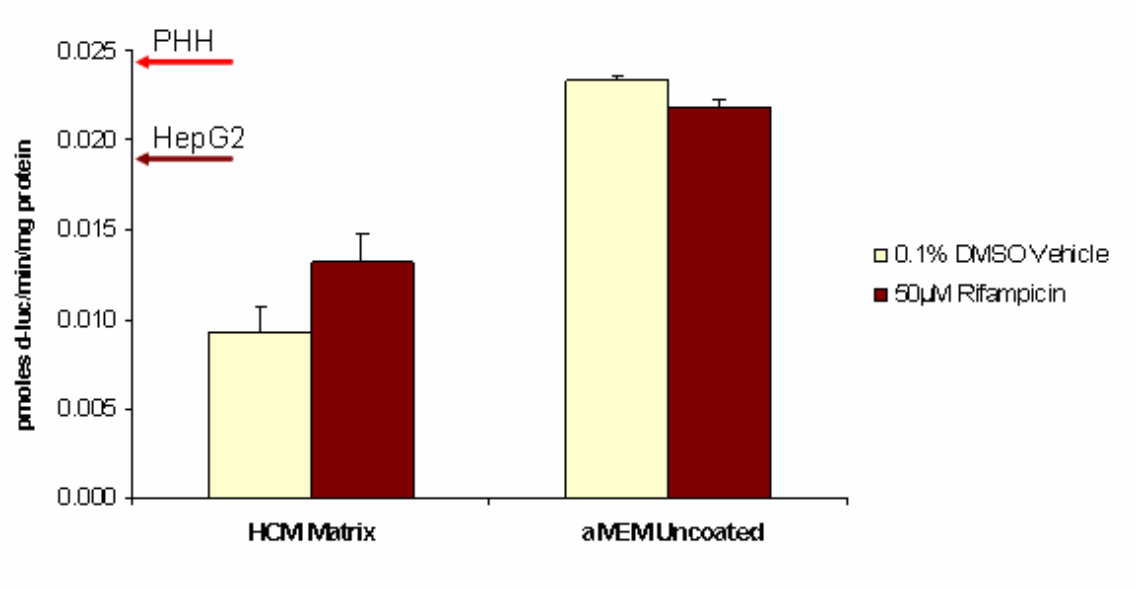


Figure A-1. CYP3A activity was compared in HC-04 cells cultured in HCM culture medium on collagen matrix or adapted to αMEM and cultured on tissue culture plastic. Activity was measured following 48 hour treatment with either 50µM rifampicin or 0.1% DMSO vehicle. Activity levels of freshly isolated PHH and HepG2 cells are indicated by arrows on the *y* axis. Indicated PHH activity was the lowest level of activity measured (n=4 +/- sd).

CYP3A Activity Decreased Following a Month in Culture

CYP3A activity was measured in unadpated or adapted HC-04 cells each week for a month. During this time a decrease in CYP3A activity was measured in all culture conditions (Figure A-2).

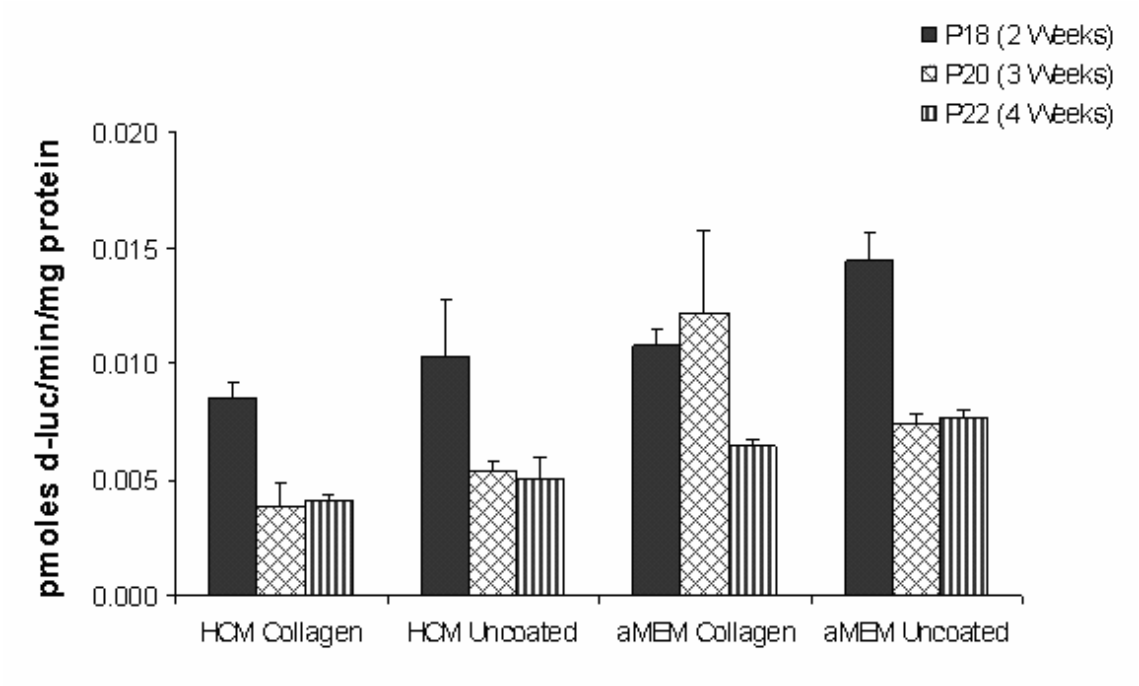


Figure A-2. CYP3A activity was measured over 4 weeks in HC-04 cells cultured in HCM or αMEM +/- collagen matrix. (n=4+/-sd).

HC-04 Cells Performed Poorly in 3D Culture

Even when encapsulated at higher seeding densities, HC-04 cells did not proliferate well in 3D culture. Spheroid formation was poor and CYP3A activity was negligible under these conditions (Figure A-3, encapsulation experiment was repeated twice, functional data not shown).

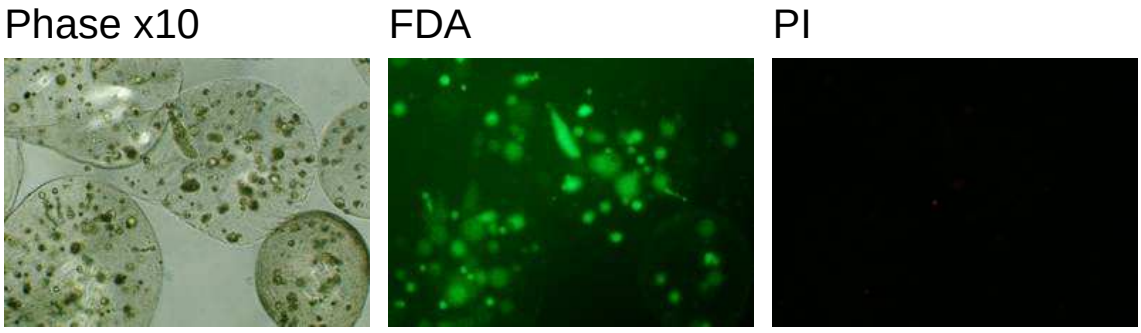


Figure A-3. Phase contrast (x10 magnification) and FDA/PI (live/dead) viability staining of alginate encapsulated HC-04 cells following 8 days of culture.

Subsequent Finding

HC-04 Cells were Infected with Mycoplasma Which Affected Cell Function

Following the initially poor results obtained with HC-04 cells, it was subsequently determined that these cells were heavily detected with mycoplasma. Furthermore it was ascertained that mycoplasma was present in a sample of the supernatant that the cells were received in originally (Figure A-4).

Gel 1
1. HepG2 cells January 2009
2. HepG2 cells encapsulated **after** contaminated HC-04 cells
3. HepG2 cells encapsulated **before** contaminated HC-04 cells
4. HC-04 supernatant sample October 2008
5. HC-04 cells January 2009
6. HC-04 frozen cells stock
7. H_20 control

Gel 2
1 -4. Samples taken from Inotech encapsulator after exposure to contaminated HC-04 cells

5. Strong positive control
6. Weak positive control
7. H_20 Control

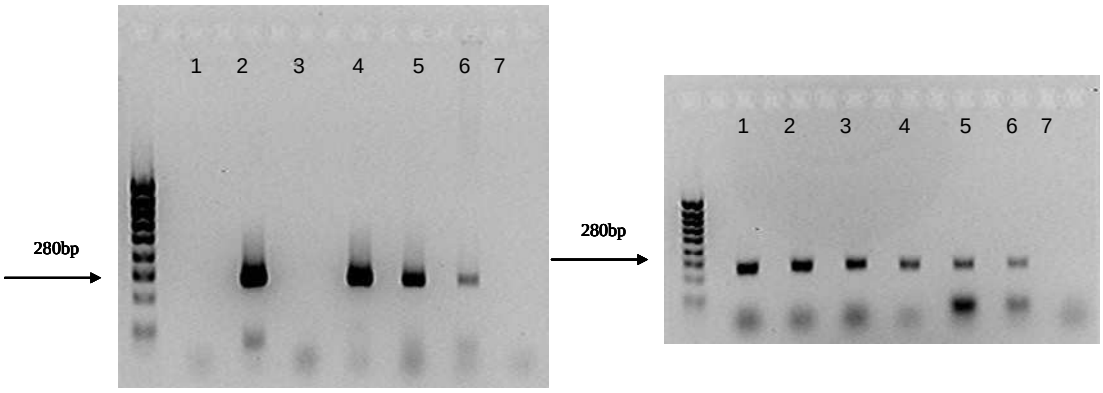


Figure A-4. Mycoplasma PCR carried out February 2009. Mycoplasma band appears at 280bp and was shown in all HC-04 cells and HepG2 cells encapsulated after contaminated HC-04 cells.

Mycoplasma contamination was not removed by ciproxin treatment, but was eliminated following 4 cycles of BM-Cyclin treatment (Roche 10 799 050 001). Cells were tested each week and remained mycoplasma free for the remaining time (Figure A-5).

28th April 2009

1. HC04
2. Caco2
3. LS147T
4. Weak positive control
5. Strong positive control
6. H_2O control

9th June 2009

1. HepG2 04/06/09
2. HCO4 08/06/09
3. HepG2 08/06/09
4. HepG2 08/06/09
5. HepG2 08/06/09
6. Weak positive control
7. Strong positive control
8. H_2O control

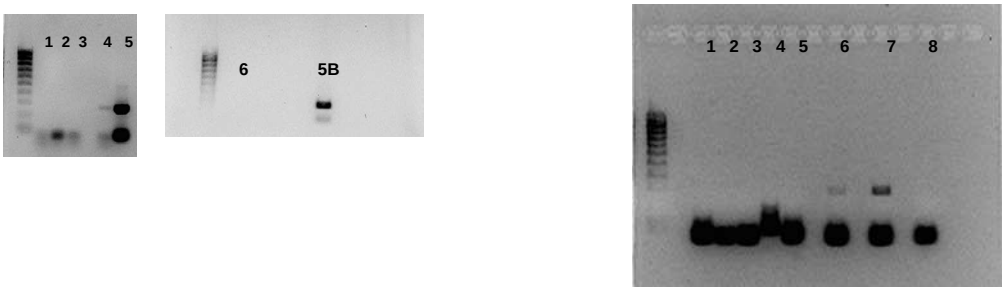


Figure A-5. (left) Mycoplasma PCR carried out on HC-04 cells following BM-Cyclin treatment and on a number of other LG cell lines. (right) Mycoplasma PCR carried out on HC-04 cells 3 months after BM cyclin treatment and on HepG2 cells obtained from 3 separate lab researchers. Mycoplasma band appears at 280bp and was shown in positive control samples only. The PCR shown in this figure was carried out by a colleague.

Due to a number of constraints, including the cost of reagents, only HC-04 cells that were adapted to LG culture conditions were treated with BM-Cyclin. Therefore it was not possible to repeat experiments 1 and 2. The subsequent functional assays of HC-04 cells demonstrated that the presence of mycoplasma had a significant effect on CYP3A function in HC-04 cells; any results obtained with the contaminated cells were discounted.

Appendix B

The Duration of Cytochrome P450 Induction in HepG2 Cells Following Removal of Inducer

The duration of CYP induction *in vitro* following removal of inducer was measured in HepG2 cells following exposure to the CYP1A inducer DBA.

Methods

Hep G2 cells were cultured in the presence of 10µM DBA for either 24 or 48 hours. After this time, culture media containing inducer was removed, cells were washed 3 times with HBSS and complete culture medium without inducer added. ECOD activity was measured either 0, 24 or 48 hours following removal of inducer. Data was normalised to total protein content, measured by the BCA assay.

Results

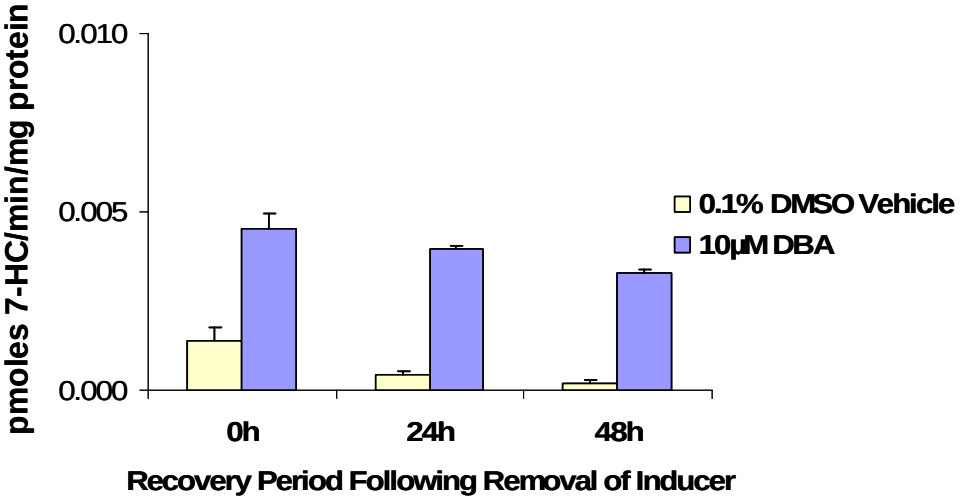


Figure B-1. DBA exposure (10µM) resulted in increased ECOD activity in HepG2 cells following a 24 hour induction. ECOD activity was then maintained in the absence of inducer and decreased gradually 0-48 hours after DBA removal.

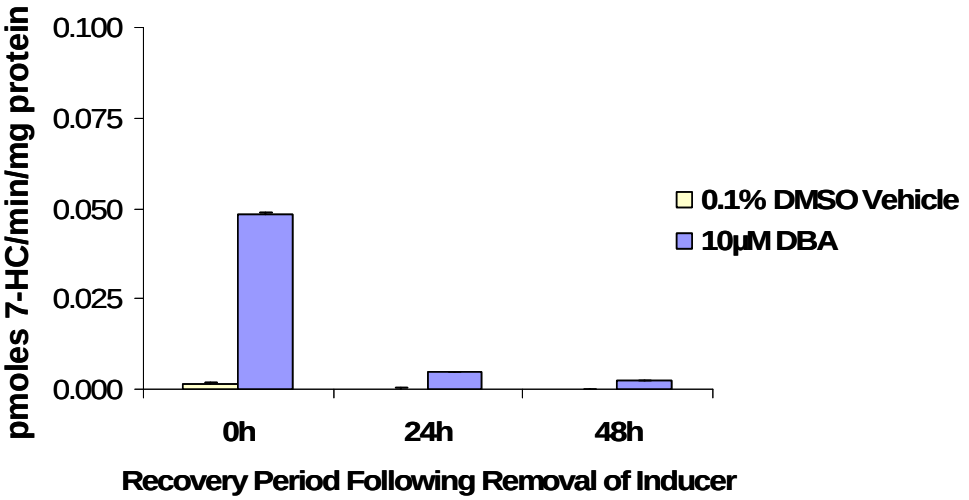


Figure B-2. There was a greater initial induction of ECOD activity following 48 hours DBA exposure, however, there was a greater decline in activity during the first 24 hours following removal of inducer.

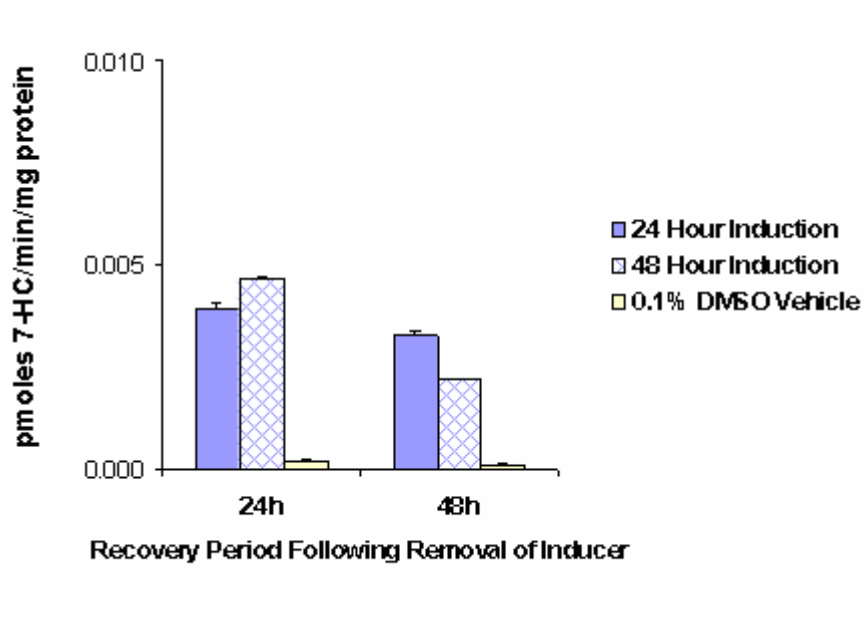


Figure B-3. Increased ECOD activity following exposure to 10µM DBA was maintained in HepG2 cells following removal of inducer. Although a 48 hour DBA incubation resulted in greater induction (seen in Figure B2), the greater decline in ECOD activity during the first 24 hours reduced ECOD activity to the level measured following a shorter initial incubation (24 hours).

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